Potential benefits of functional amino acids in fish nutrition

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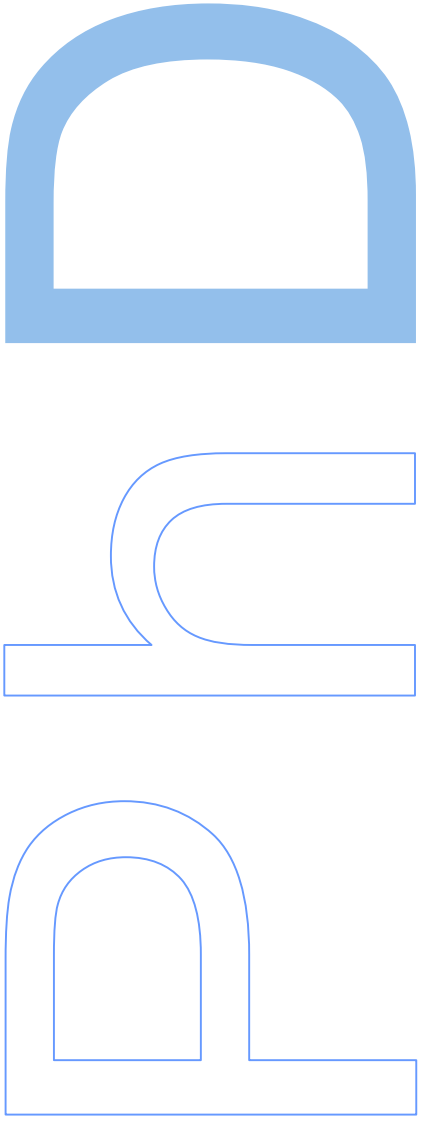

Nota prévia

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Em todas as publicações decorrentes deste trabalho é devidamente referido que as instituições de origem do doutorando Filipe Fernandes Coutinho são:

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Abstract

Replacing fish meal (FM) in aquafeeds by more sustainable ingredients, such as vegetable protein sources, remains crucial for the sustainable development of aquaculture. However, incorporating high levels of plant feedstuffs (PF) in aquafeeds for carnivorous fish, without compromising fish performance and health, still represents a challenge. Therefore, it is urgent to develop sustainable and effective strategies to mitigate the effect of these constrains. One of these strategies is the use of potential functional nutrients, namely amino acids (AA), to improve fish health and production.

Growing evidences reveal that some AA play important roles in the modulation of fish somatic growth, nutrient retention efficiency, intestinal health and antioxidant defence. In this context, the present work aimed to evaluate the potential benefits of supplementing four functional AA - glutamine (Gln), arginine (Arg), methionine (Met) and taurine (Tau) in PF-based diets for gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*), the two main marine fish species produced in southern Europe aquaculture. To evaluate the effects of dietary AA supplementation a holistic approach was used, including the analysis of fish growth performance, feed utilization, whole-body composition, intestinal nutrient absorption capacity, liver and intestine AA metabolism, and oxidative status.

In Chapters 2, four isolipidic (18% lipid) and isonitrogenous (43% protein) diets, incorporating 50/50 of FM and PF protein, were supplemented with 0, 0.5, 1 and 2% Gln, and tested on a 6 weeks growth trial with gilthead sea bream juveniles (mean weight 13.0 g). Zootechnical performance, body composition, intestinal nutrient absorption capacity, hepatic and intestinal AA catabolism and Gln metabolism were unaffected by dietary Gln supplementation. However, dietary Gln supplementation increased glutathione (GSH) content of liver and intestine, and superoxide dismutase (SOD) activity in the intestine, but without affecting the overall oxidative damage. Data on hepatic and intestinal Gln metabolism suggested higher Gln use for energy production by the intestine than by the liver. The comparison between hepatic and intestinal antioxidant enzymes activities and GSH content seem to reflect a higher intestinal dependency of GSH in the antioxidant response. Overall, clear differences between liver and intestine Gln metabolism and antioxidant response were observed and dietary Gln supplementation was shown to modulate gilthead sea bream's antioxidant response.

In Chapter 3, four diets similar to those used in Chapter 2, but supplemented with 0, 0.5, 1 and 2% Arg, were tested on a 6 weeks growth trial with gilthead sea bream juveniles

(mean weight 13.0 g). Fish growth performance, whole-body composition, intestinal nutrient absorption capacity, hepatic and intestinal AA catabolism were not affected by dietary Arg supplementation. These results suggest that, within the levels tested, no toxicity of dietary Arg excess or Arg-Lysine antagonism occurred. Dietary Arg surplus modulated the enzymatic antioxidant response in both liver and intestine, but did not affect the overall oxidative status. Data on hepatic and intestinal antioxidant enzymes activities corroborate the hypothesis raised in Chapter 2 of higher intestinal dependency of GSH in the antioxidant response. These results indicate that, similarly to what was observed with Gln (Chapter 2), dietary Arg supplementation has limited potential to enhance gilthead sea bream performance, but seems to modulate hepatic and intestinal antioxidant defences.

The last study (Chapter 4) investigated the potential of Met and Tau supplementation to improve European sea bass juveniles (mean weight 6.9 g) growth performance, feed utilization, and oxidative status. For that purpose, four isolipidic (18%) and isonitrogenous (43% protein) diets containing 82% of the protein from plant origin were formulated to have a Met level 12% below or 15% above requirement, and supplemented or not with 1% Tau. European sea bass juveniles performed well with the unsupplemented diet and further supplementation with Met and/or Tau did not affect fish growth performance, feed utilization and whole-body composition. These results seem to indicate that European sea bass juveniles were capable of synthesising Tau at a sufficient rate to meet requirement, and that the established Met requirement for European sea bass may be over-estimated and should be re-evaluated taking into account total dietary sulphur AA level of practical plant-protein-rich diets.

In the liver, dietary Met supplementation enhanced hepatic antioxidant enzymes activities whereas the opposite occurred with dietary Tau supplementation. Moreover, in the intestine Met supplementation increased GSH content, while the opposite was observed with Tau supplementation. Still, neither dietary Met nor Tau supplementation had noticeable effects on liver and intestine oxidative damage. Thus, the general decrease of antioxidant enzymes activities and of GSH levels observed with dietary Tau supplementation seems to indicate that Tau exerts an antioxidant action by avoiding reactive oxygen species generation. Indeed, glutathione peroxidase and glutathione reductase activities in the intestine were higher with the low Met - low Tau diet and decreased with the dietary Met or Tau supplementation, attesting the important antioxidant roles of these AA.

Overall, this work indicates that dietary Gln or Arg supplementation in gilthead seabream, and Met and Tau supplementation in European sea bass, have limited potential to

enhance fish growth performance, but seem to modulate fish hepatic and intestinal antioxidant defences. Present work also provides evidence that European sea bass can be produced with high-plant-protein diets with a Met level below the established requirement for the species and without Tau supplementation. Considering the low Met level but high total sulphur containing AA in PF, a re-evaluation of Met and total sulphur AA requirements of European sea bass and, eventually other species, seems important, particularly when considering the use of novel plant-feedstuffs based diets.

Keywords: Gilthead sea bream; European sea bass; Fish nutrition; Intestinal nutrient absorption capacity; Amino acids catabolism; Oxidative status.

Resumo

A substituição da farinha de peixe (FP) nas rações para peixes, por ingredientes mais sustentáveis, tais como fontes proteicas vegetais, continua a ser crucial para o desenvolvimento sustentável da aquacultura. Contudo, a incorporação de níveis elevados de matérias-primas vegetais (MPV,) nas rações para peixes carnívoros sem comprometer a sua performance e saúde, ainda representa um desafio. É, portanto, urgente desenvolver estratégias, sustentáveis e efetivas, para mitigar o efeito dessas restrições. Uma dessas estratégias é o uso de potenciais nutrientes funcionais, nomeadamente os aminoácidos (AA). Um crescente número de evidências associa os AA a importantes funções reguladoras do crescimento somático dos peixes, da eficiência de retenção dos nutrientes, da saúde intestinal e das defesas antioxidantes. Neste contexto, o presente trabalho pretendeu avaliar os potenciais benefícios de quatro AA funcionais - glutamina (Gln), arginina (Arg), metionina (Met) e taurina (Tau) - quando suplementados em dietas maioritariamente vegetais, para a dourada (*Sparus aurata*) e o robalo Europeu (*Dicentrarchus labrax*), as duas principais espécies de peixes marinhos produzidas em aquacultura no Sul da Europa. Usou-se uma abordagem holística para avaliar o efeito da suplementação destes AA, no crescimento, na utilização do alimento, na composição corporal, na capacidade intestinal de absorção de nutrientes, no metabolismo dos AA no fígado e no intestino e no estado oxidativo dos peixes.

No Capítulo 2, quatro dietas com igual conteúdo de lípidos (18%) e de proteína (43% de proteína), com metade da proteína fornecida por FP e a outra metade por MPV, e suplementadas com 0, 0,5, 1 e 2% de Gln, foram testadas num ensaio de crescimento de 6 semanas com juvenis de dourada (peso médio de 13,0 g). O crescimento, a composição corporal, a capacidade intestinal de absorção de nutrientes, o catabolismo de AA e o metabolismo da Gln, no fígado e no intestino, não foram afetados pela suplementação das dietas com Gln. Ainda assim, a suplementação das dietas com Gln aumentou o conteúdo de glutationa (GSH) no fígado e no intestino e a atividade da enzima superóxido dismutase (SOD) no intestino, sem contudo afetar o dano oxidativo. Os dados do metabolismo da Gln no fígado e no intestino sugeriram um maior uso da Gln para a produção de energia no intestino do que no fígado. Enquanto a comparação entre a atividade enzimática antioxidante hepática e intestinal e dos conteúdos de GSH pareceram revelar uma maior dependência da GSH por parte da resposta antioxidante intestinal. Em suma, foram elucidadas algumas diferenças claras entre o metabolismo

da Gln e da resposta antioxidante no fígado e no intestino e demonstrou-se que a suplementação das dietas com Gln modelou a resposta antioxidante da dourada.

No Capítulo 3, quatro dietas com a mesma formulação que as dietas usadas no Capítulo 2, mas suplementadas com 0, 0,5, 1 e 2% de Arg, acima das necessidades, foram testadas num ensaio de crescimentos de 6 semanas em juvenis de dourada (peso médio de 13,0 g). O crescimento, a composição corporal, a capacidade intestinal de absorção de nutrientes e o catabolismo de AA no fígado e no intestino não foram afetados pela suplementação das dietas com Arg. Estes resultados sugerem que, nos níveis testados, não ocorreu qualquer efeito tóxico resultante do excesso de Arg ou de um antagonismo entre a Arg e a lisina. O nível de Arg nas dietas modelou a resposta enzimática antioxidante do fígado e do intestino, mas não afetou o dano oxidativo. Os dados das atividades enzimáticas antioxidantes do fígado e do intestino corroboram a hipótese proposta no Capítulo 2 de uma maior dependência da resposta antioxidante intestinal pela GSH. Estes resultados indicam que, de forma semelhante à Gln (Capítulo 2), a suplementação das dietas com Arg teve um reduzido potencial para aumentar o crescimento da dourada, mas pode modelar as defesas antioxidantes do fígado e do intestino.

O último estudo (Capítulo 4) investigou o potencial da suplementação com Met e Tau para melhorar o crescimento, a utilização do alimento e o estado oxidativo de juvenis de robalo Europeu (7 g de peso médio). Para isso, formularam-se quatro dietas à base de MPV, com 82% da proteína fornecida por MPV, e igual conteúdo de lípidos (18%) e de azoto (43% de proteína), de forma a terem um nível de Met 12% abaixo ou 15% acima das necessidades, e suplementados ou não com 1% de Tau. Os juvenis de robalo Europeu cresceram bem com a dieta sem suplementação de Met ou Tau e o aumento dos níveis de Met e/ou Tau na dieta não afetou o crescimento, a utilização do alimento ou a composição corporal. Estes resultados parecem indicar que os juvenis de robalo Europeu têm a capacidade de sintetizar Tau, a uma taxa suficiente para cobrir as necessidades, e que as necessidades de Met podem estar sobrestimadas, necessitando de ser reavaliadas, tendo em consideração os níveis de cistina de dietas práticas à base de MPV.

No fígado, enquanto a suplementação das dietas com Met levou ao aumento da atividade enzimática antioxidante, a suplementação com Tau teve maioritariamente o efeito contrário. Para além disso, no intestino, a suplementação com Met aumentou o conteúdo de GSH, enquanto a suplementação com Tau teve o efeito oposto. Ainda assim, o dano oxidativo do fígado e do intestino não foram afetados pela suplementação das dietas com Met e/ou Tau. Neste sentido, os efeitos maioritariamente de redução da

atividade enzimática e do conteúdo em GSH observados com o aumento do nível de Tau na dieta parecem indicar que a ação antioxidante de Tau se ficará a dever a uma redução da produção de espécies reativas de oxigénio. Para além disso, a atividade das enzimas glutationa peroxidase e glutationa redutase no intestino foram maiores para a dieta com baixos níveis de Met e Tau e diminuiu com a suplementação de Met ou Tau, revelando o importante papel antioxidante destes AA.

Em suma, este trabalho indica que a suplementação de dietas para dourada com Gln ou Arg, e de dietas para robalo Europeu com Met e/ou Tau apresentam um potencial limitado de aumentar o crescimento dos peixes, mas podem ainda assim modelar as defesas antioxidantes do fígado e do intestino. O presente trabalho fornece ainda evidências de que o robalo Europeu pode ser produzido com uma dieta rica em proteínas de origem vegetal, com um nível de Met 12% abaixo das necessidades estimadas para esta espécie e sem suplementação com Tau. Considerando os reduzidos níveis de Met mas elevados níveis de AA contendo enxofre das MPV, parece importante efetuar uma reavaliação das necessidades de Met e de AA contendo enxofre no robalo Europeu, e eventualmente de outras espécies, particularmente considerando o uso de novas dietas à base de MPV.

Palavras-chave: Dourada; Robalo Europeu; Nutrição de peixes; Capacidade de absorção intestinal de nutrientes; Catabolismo de aminoácidos; Estado oxidativo.

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List of Abbreviations

AA - amino acids

ADP - adenosine diphosphate

Ala - alanine

ALAT - alanine aminotransferase

ANF - anti-nutritional factors

Arg - arginine

ASAT - aspartate aminotransferase

Asn - asparagine

Asp - aspartate

ATP - adenosine triphosphate

BAP - biological antioxidant potential

BBM - brush border membrane

BBMV - brush border membrane vesicles

BSE - bovine spongiform encephalopathy

CAT - catalase

CDO - cysteine dioxygenase

CIAA - conditionally indispensable amino acids

cm - centimeter

CPS III - carbamoyl phosphate synthetase III

CSD - cysteine sulfinic acid decarboxylase

Cys - cysteine

DAA - dispensable amino acids

DHA - docosahexaenoic acid

DNA - deoxyribonucleic acid

EPA - eicosapentaenoic acid

ET – enzyme-treated

EU - European Union

FAA - free AA

FM - fishmeal

FO - fish oil

g - gram

G6PD - glucose-6-phosphate dehydrogenase

GCN2 - general control nonderepressible 2

GDH - glutamate dehydrogenase

GH - growth hormone

Gln - glutamine

GlNase - phosphate-activated glutaminase

Glu - glutamate

Gly - Glycine

GPX - glutathione peroxidase

GR - glutathione reductase

GSase - glutamine synthetase

GSH - reduced glutathione

GSSG - oxidized glutathione

GTP - guanidine triphosphate

H_2O_2 - hydrogen peroxide

His - histidine

HUFA - highly unsaturated fatty acids

IAA - indispensable amino acids

IGF-1 - insulin-like growth factor 1

IgM - immunoglobulin M

Ile - isoleucine

kg - kilograms

Leu - leucine

LPO – lipid peroxidation

Lys - lysine

m - meter

Met - methionine

mg - milligram

MHA - met hydroxyl analogue

MSR - methionine sulfoxide reductase

mRNA - messenger ribonucleic acid

mTOR - mammalian target of rapamycin

NAD+ - nicotinamide adenine dinucleotide

NADH - nicotinamide adenine dinucleotide hydrate

NADPH - nicotinamide adenine dinucleotide phosphate

NH_4^+ - ammonium cation

NO - nitric oxide

NO_2 - nitrogen dioxide

NSP - non-starch polysaccharides

$O_2^{-\cdot}$ - superoxide radical

$ONOO^-$ - peroxynitrite

PAP - processed animal proteins

PepT1 - peptide transporter 1

PF - plant feedstuffs

Phe - phenylalanine

Pro - proline

PSA - phosphoserine aminotransferase

PUFA - polyunsaturated fatty acids

RNS - reactive nitrogen species

ROS - reactive oxygen species

SAM - s-adenosylmethionine

SDG - sustainable development goals

Ser - serine

SOD - superoxide dismutase

SE - solvent-extracted

t - tonnes

Tau - taurine

tGSH - total glutathione

Thr - threonine

TOR - target of rapamycin

Trp - tryptophan

TSAA - total sulphur AA

Tyr - tyrosine

Val – valine

·OH - hydroxyl radical

°C - degrees Celsius

$^{1}O_{2}$ - singlet oxygen

Chapter 1

General Introduction

1.1. Aquaculture development and population growth

In the last five decades world supply of fish for human consumption grew at an average annual rate of 3.2% outpacing the 1.6% growth rate of world population. In fact from 2001 to 2013 world *per capita* fish consumption increased 22%, from 16.1 kg to 19.7 kg, reaching a new record high of 20 kg in 2014. In global terms the contribution of fish consumption to the population intake of animal protein is also increasing, reaching 17% in 2013 (EUMOFA, 2016; FAO, 2016).

From 2013 to 2014 apparent consumption of fish products in the European Union (EU) increased 409.000 tonnes, reaching almost 13 million tonnes. This represents a 3.5% increase of the average *per capita* fish consumption in the EU, which reached 25.53 kg (EUMOFA, 2016). The rise in consumption was more significant for aquaculture products, with a 6% increase, while wild fish consumption, despite representing 75% of total consumption, increased only 2.7%. However, the *per capita* fish consumption within the EU is very variable, ranging from 5 kg in Serbia and Albania to 90 kg (values for 2014) in Iceland, which is the highest in the world. For the same period Portugal *per capita* fish consumption reached 53 kg, being the second highest in the EU and almost certainly third in the world behind Japan (EUMOFA, 2016; FAO, 2015, 2016).

The continuous growth of world population, the rapid urbanization and rising living standards, as well as the technological developments in food processing, packaging, and distribution and the increasing recognition of fish as nutritious food are expected to support the continuous increase of apparent *per capita* fish consumption. In fact, in developed countries fish is increasingly considered to be a nutritious food that should be included in a healthy diet due to its high quality proteins, with adequate amino acids (AA) profile, essential fats (e.g. long-chain omega-3 fatty acids), vitamins (D, A, and B) and minerals (including calcium, iodine, zinc, iron, and selenium) (EUMOFA, 2016; FAO, 2015, 2016).

With capture fishery production relatively static at 90-95 million tonnes since mid-1990s the additional demand of fisheries products has been fulfilled by aquaculture production (Figure 1A). This trend is expected to continue and it is anticipated that aquaculture will remain the fastest-growing sector for animal food production by 2025, despite a decline in its annual growth rate from 2.5% in the previous decade, to 1.5% by 2025. Yet, the share of aquaculture to total fishery production is expected to grow from 44% in 2013–15 to 52% in 2025 (Figure 1B).

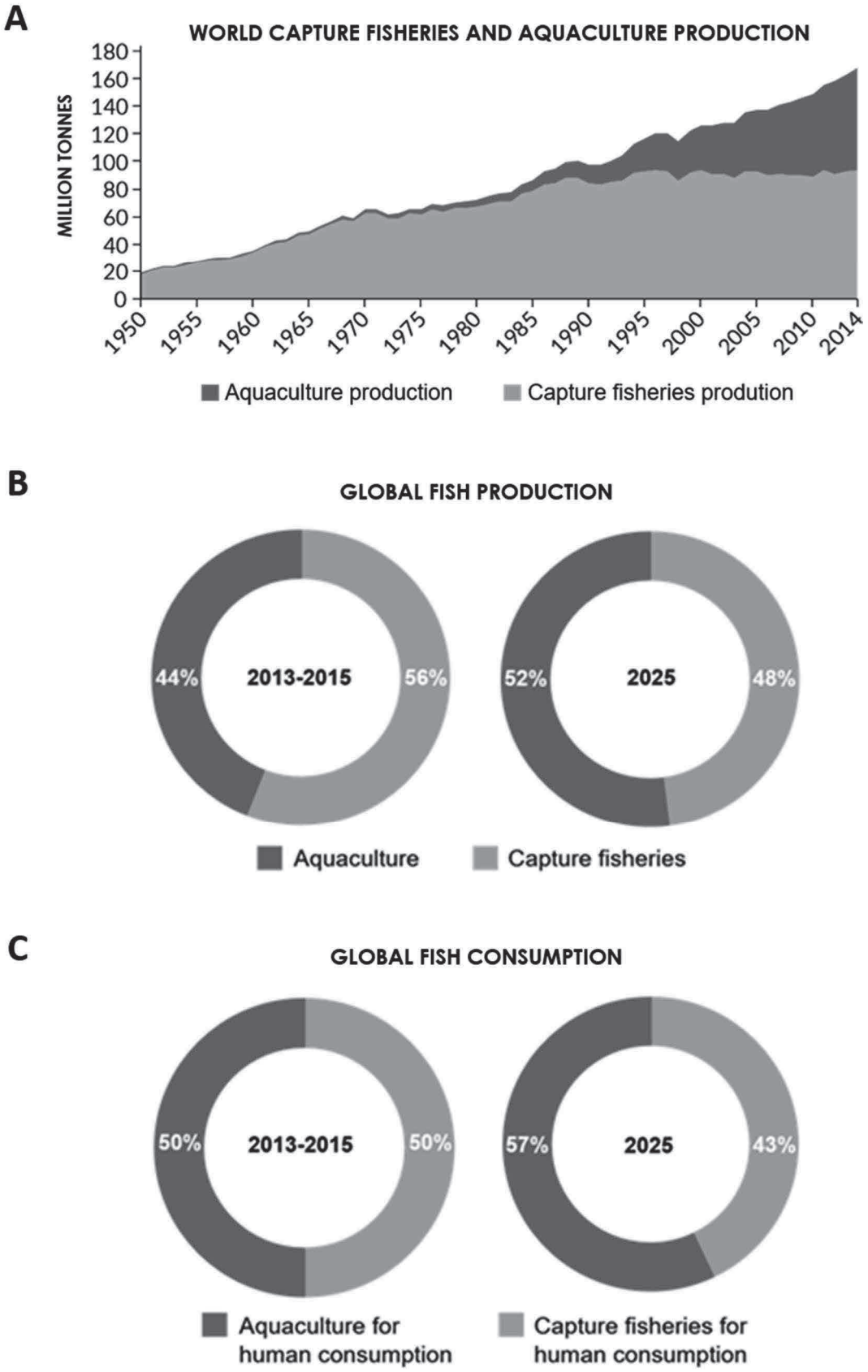

Figure 1. Aquaculture developments: current status, trends and future prospects. A: World capture fisheries and aquaculture production; B: Relative share of aquaculture and capture fisheries in global production; C: Relative share of aquaculture and capture fisheries in global consumption (adapted from FAO, 2016).

However, as almost all fish produced from aquaculture are destined for human consumption, the share of aquaculture in global fish consumption is anticipated to increase from 50% in 2013-2015 to 57% in 2025 (Figure 1C). It is however important to note that the share of aquaculture production varies widely among continents, accounting for only 18% of total fish production in Europe in 2014 (FAO, 2016).

The United Nations expect world population to reach 8.5 thousands of millions in 2030 and 9.7 billion in 2050. Under a context of climate change, economic and financial uncertainty, and limited natural resources, feeding 9.7 billion people in 2050 is considered one of the greatest challenges of the twenty-first century and motivated the United Nations Member States, on 25 September 2015, to adopt the 2030 Agenda for Sustainable Development and the Sustainable Development Goals (SDGs). Among other objectives, this Agenda aims to ensure the sustainable development of fisheries and aquaculture in social, economic and environmental terms, with the SDG 14 expressly focussing on the oceans and marine environments (FAO, 2016).

1.2. Towards sustainable aquafeeds

As a consequence of past decades stagnated fisheries and increased demand for fisheries products, the share of world fish production supplied for non-food products decreased from 33% in the 1960's to 13% in 2014, with fishmeal (FM) and fish oil (FO) representing 76% of this total in 2014. With 58.1% of worlds' marine fish stocks fully exploited in 2013, and no additional improvement being anticipated, especially regarding pelagic catches, any increase in FM and FO production will have to come from recycling of by-products with, however, some likely low impact in the overall products composition (FAO, 2016).

FM and FO are still considered the most nutritiously balanced ingredients for carnivorous fish aquafeeds, due to their high palatability and digestibility and ideal fatty acids and AA profiles. However, with the progressive decline in production and rising prices of such commodities, it has become imperative to find more affordable and sustainable protein and lipid sources to partially or completely replace FM and FO (Oliva-Teles *et al.*, 2015). In that process, FM and FO have become more strategic ingredients, being included at high concentrations only at specific production stages (e.g. starter, finishing and broodstock feeds) (ARRAINA, 2015). However, these changes in feeds formulation are especially challenging for carnivorous fish given that, compared to other animals, they have higher dietary protein and AA requirements, are more dependent on dietary protein

rather than on glucose or lipids to meet their metabolic needs and tolerate lower dietary carbohydrate levels (Halver and Hardy, 2002). Besides, fresh water and marine fish species have different essential fatty acids requirements, and because of that marine fish are dependent on FO to supply the required long-chain omega-3 fatty acids (Oliva-Teles et al., 2015).

Protein is the most expensive component of fish feeds, justifying the large body of research focussed on reducing aquafeeds dependency of the unsustainable and ever more costly FM. In the last decade some relevant progress was made in the reduction of dietary FM dependency with, however, very distinct results between carnivorous fish (with high protein requirements, 40 to 55% of the diet) and herbivorous and omnivorous fish (with lower protein requirements, 25 to 35% of the diet) (Wilson, 2002; NRC, 2011; Lall and Dumas, 2015). Currently, diets for herbivorous and omnivorous fish are almost devoid of FM, having soybean meal as the main protein source. On the other hand, diets for marine fish and salmonids still include around 20% and 15-18% FM, respectively (Tacon and Hasan, 2011; IFFO, 2013).

One of the main issues with the use of alternative protein sources is their distinct AA profile when compared to FM. Like other animals, fish do not have a true protein requirement but instead have a requirement for a well-balanced mixture of indispensable amino acids (IAA), and dispensable amino acids (DAA; Table 1). IAA are either not biosynthesized or insufficiently biosynthesized and therefore must be supplied by the diet. On the other hand, DAA are efficiently synthesised *de novo* by the organism from an amino source and an α-keto acid precursor, and therefore do not need to be supplied by the diet, though the molecules required for their synthesis still need to be provided. Among the AA that are synthesised from other IAA, some are considered conditionally indispensable amino acids (CIAA; Table 1), if under some circumstances their metabolic demand may rise above the biosynthetic capacity of the organism (Wilson, 2002; Li *et al.*, 2009; NRC, 2011).

At present plant feedstuffs (PF) and, to a lesser extent, processed animal proteins (PAP) constitute the main alternative protein sources to FM. Compared to PF, PAP, such as poultry meal, feather meal, and blood meal, present higher nutritional value for fish due to the absence of anti-nutritional factors (ANF), good palatability, high protein content (60 to 90%), and appropriate IAA content, with the exception of methionine (Met) (ARRAINA, 2015).

Table 1. Nutritionally indispensable, dispensable and conditionally indispensable AA for fish.

Indispensable AA	Dispensable AA	Conditionally indispensable AA*
Arginine	Alanine	Cysteine
Histidine	Asparagine	Glutamine
Isoleucine	Aspartate	Hydroxyproline
Leucine	Glutamate	Proline
Lysine	Glycine	Taurine
Methionine	Serine	Tyrosine
Phenylalanine		
Threonine		
Tryptophan		
Valine		

Adapted from: Li *et al.* (2009).
*depending on diet formulation, species, age, physiological and environmental factors, stress condition and disease status.

Even though PAP present high saturated fat and ash levels, which limits their inclusion in aquafeeds, they have been considered the most promising alternative ingredients to FM. In fact, their inclusion in fish feeds was recently enhanced, namely in European countries, following the re-authorization of the use of PAP from non-ruminants in aquafeeds, from the 1st of June 2013. Despite this increasing trend of PAP use in compound feeds and their competitive prices, PAP still represents less than 1% of total feedstuffs used in global aquafeeds production. Consumers concerns regarding the potential risk of disease transmission, associated to the recent problem of bovine spongiform encephalopathy (BSE), inevitably presents some constrains to their inclusion in fish feeds. As well, the few studies available in Europe regarding the suitability and maximum inclusion levels of the newly produced PAP as FM alternatives, and the current wide use of PF in aquafeeds, are expected to favour the use of PF in aquafeeds formulation in Europe (Karapanagiotidis, 2014; Lall and Dumas, 2015).

A number of PF are highly available in global market. PF present relatively constant nutritional composition, competitive prices and few food safety concerns, making them suitable for incorporation in aquafeeds (NRC, 2011; Olsen and Hasan, 2012; Oliva-Teles *et al.*, 2015). The main plant protein sources used in compound feeds for aquaculture include oilseeds (soybean meal, rapeseed meal, cottonseed meal, and sunflower seed meal); legumes (peas, lupins, and faba beans); cereals (wheat, rice, maize, barley, and

corn); and plant protein concentrates from different PF (Tacon and Hasan, 2011; Oliva-Teles *et al.*, 2015).

In the down side, PF present a highly variable protein content; high carbohydrate contents, both digestible (such as starch) and non-digestible (such as fibres); IAA inadequacies (exception made for soy protein concentrate or potato protein concentrate); a diversity of ANF including phytate that limits the availability of dietary phosphorus (NRC, 2011; Tacon and Hasan, 2011; ARRAINA, 2015). Moreover, as PF are less palatable than FM or PAP additional care must be taken when formulating high plant protein based diets in order not to compromise fish feed intake. This is usually achieved by the inclusion of feed attractants such as squid meal, fish protein hydrolysates, and free AA (FAA), depending on fish species (Dias *et al.*, 1997; Kader *et al.*, 2010; Kader *et al.*, 2012; ARRAINA, 2015).

Soybean meal is the main plant feedstuff used in aquafeeds and it is also the most available oilseed worldwide. Oilseeds have competitive prices when compared to FM (Shepherd and Jackson, 2013) but present moderate protein content (38 to 52%), ANF and IAA deficiencies, with Met being the first limiting AA in soybean meal and lysine (Lys) in sunflower and cottonseed meals. As a result of their low protein content, oilseeds generally replace up to 20-40% of FM protein, or 15-30% of the diet, although for carnivorous fish dietary incorporation is usually limited to 10-20% (Tacon and Hasan, 2011).

Legumes also present low protein contents (22 to 30%), have ANF including non-starch polysaccharides (NSP), and may replace 10 to 30% of FM protein, being generally incorporated at relatively low levels in carnivorous fish diets (10 to 15% of the diet) (Medale and Kaushik, 2009; Tacon *et al.*, 2009; Oliva-Teles *et al.*, 2015).

Cereals are the most affordable PF but present the lowest protein content (9 to 12%), are deficient in Lys and are rich in starch (up to 60%). For those reasons, cereals incorporation in carnivorous fish diets is low (10 to 20%), contributing to about 5% of dietary protein (Tacon *et al.*, 2009; Oliva-Teles *et al.*, 2015).

On the other hand, plant protein concentrates, namely corn and wheat glutens and the less abundant soy, pea, potato, and rapeseed protein concentrates, have high protein content (60 to 80%) and are almost devoid of ANF, making them the most nutritionally suitable PF alternatives to FM. Even though, they may have some IAA deficiencies, with Lys, threonine (Thr) and Met as the first limiting AA, their high price (except corn gluten) is the main factor limiting their increased use in aquafeeds. Although it is known that plant protein concentrates may replace 30 to 100% of dietary FM protein, which

corresponds to 10 to 60% dietary incorporation, commercial diets for carnivorous fish generally include less than 15% plant protein concentrates, with the exception of corn gluten, which incorporation may ascends to 20-25% (Gatlin *et al.*, 2007; Tacon and Hasan, 2011; Oliva-Teles *et al.*, 2015).

When attempting to replace FM by PF in aquafeeds caution must be taken not to compromise fish intestine homeostasis, disease resistance and health. This is particularly challenging in carnivorous marine fish and salmonids, since these species are not naturally prepared to utilize PF, digest their cell-wall components, and to use their high carbohydrate contents. Plant-rich diets may compromise fish digestion and feed utilization, while the presence of ANF may reduce AA utilization and induce some pathological effects (Buddington *et al.*, 1997; Panserat and Kaushik, 2002; Gatlin *et al.*, 2007). For instance, Atlantic salmon (*Salmo salar*) is known to develop the so-called soybean-induced enteritis even when fed diets with low soybean inclusion levels (Krogdahl *et al.*, 2010). In marine fish, such as European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*), these negative effects related to dietary soybean appear, however, to be less pronounced (Couto *et al.*, 2014; Couto *et al.*, 2015). Meanwhile, several strategies have been developed to neutralize PF' ANF and to increase phosphorus and starch availability. These include heat processing, solvent extraction and dehulling, and using exogenous enzymes such as phytases (Glencross *et al.*, 2007; Krogdahl *et al.*, 2010). Despite such advances, the high protein requirements of salmonids and marine fish imply that only some oilseeds (soybean meal and cottonseed meal) and the more expensive plant protein concentrates are considered proper alternative protein sources to FM (ARRAINA, 2015; Oliva-Teles *et al.*, 2015).

Since the IAA profile of alternative plant ingredients are usually unbalanced for most fish species, being limiting in at least one IAA, it is usually necessary to supplement plant-based diets with the limiting IAA in order to balance IAA requirements (Fournier *et al.*, 2004; Alam *et al.*, 2011). Commercially available feed-grade AA are still limited to a few IAA, namely Lys, Met, Thr, tryptophan (Trp) and valine (Val) (NRC, 2011), and they are now widely used in the aquafeeds industry due to their competitive prices. Some care must however be taken in order to delay the intestinal absorption of these FAA, in order to guarantee the simultaneous absorption and availability for metabolism of both protein-bound and FAA. This delay of FAA intestinal absorption may be achieved by coating FAA with agar, which also helps in avoiding AA leaching into the water (Cho *et al.*, 1992; Fournier *et al.*, 2002; Peres and Oliva-Teles, 2005; Li *et al.*, 2009; Pérez-Jiménez *et al.*, 2014).

Even though Lys and Met are generally the first limiting IAA of PF used in aquafeeds, each feedstuffs is limiting in one of these IAA, allowing to adjust the aquafeed IAA profile by combining different feedstuffs, thus limiting the need for supplementing diets with feed-grade AA (Naylor *et al.*, 2009; Oliva-Teles *et al.*, 2015). Several studies have indeed highlighted that the combination of PF is a more adequate strategy for replacing FM protein than the use of single PF (Espe *et al.*, 2006; Kaushik and Seiliez, 2010; Oliva-Teles *et al.*, 2015)

Although it is now possible to completely replace FM by mixtures of alternative PF, if duly supplemented with limiting IAA, without compromising fish growth performance, until recently, little attention has been given to the impact of modifying dietary AA profile in several aspects of fish health or overall metabolism (Li *et al.*, 2009; Kiron, 2012). Impelled by the growing availability of feed-grade AA and the increased awareness of AA role in the modulation of fish somatic growth, nutrient retention, gut health, immune response and antioxidant defence, a great deal of research is now being driven to the role of AA as functional ingredients (Li *et al.*, 2009).

1.3. Protein digestion and absorption of amino acids and peptides

Animal growth and development implies the synthesis of new proteins and polypeptides, which is dependent on the regular provision of AA resultant from the digestion and absorption of dietary proteins. Regardless of the variability of the digestive tract anatomy among fish species, associated to differences in fish feeding habits, the gastrointestinal tract of fish hydrolyses proteins to tri- or di-peptides and FAA, which are then absorbed and distributed through the blood for the different organs and tissues (Clements and Raubenheimer, 2006; Wu, 2009; Wu *et al.*, 2014).

Nutrient uptake occurs primarily at the anterior end of the post-gastric intestinal region (proximal intestine), where many fish species have an extended intestinal surface area by presenting numerous pyloric caeca. Pyloric caeca increases the digestive and absorptive surface area of an otherwise relatively short intestinal tract, and are of major importance for enzymatic breakdown and absorption of feed constituents (Buddington and Diamond, 1987). At its posterior end, the intestine generally presents a distal region (distal intestine) separated by a more or less distinctive morphological aspect, where nutrient absorption capacity is reduced but where small proteins and peptides may be absorbed by endocytosis, which has both nutritional and immunological importance (Clements and Raubenheimer, 2006).

As in higher vertebrates, an absorptive epithelium forms a barrier between the interior and exterior intestinal medium of fish. Such epithelium consists mainly of mucus-secreting goblet cells and enterocytes, the cells responsible by nutrient uptake. At the luminal surface, enterocytes are joined together at the apical end of the lateral surface by junctional complexes and present numerous extensions of the epithelial layer, called microvilli, which increase the overall absorptive area and activity of membrane-bound digestive enzymes. Contiguous enterocytes form the apical surface of the epithelium known as the brush border membrane (BBM). The absorption of nutrients across the BBM of the intestinal epithelial cells stands as an important step in digestion (Ferraris and Ahearn, 1984; Bakke-McKellep *et al.*, 2000; Clements and Raubenheimer, 2006).

1.3.1. Amino acids and peptides absorption through the BBM

In fish, as in other vertebrates, di- and tripeptides and AA resultant from the hydrolysis of dietary proteins can be directly absorbed by intestinal epithelial cells. At the BBM level the transport of a large variety of di- and tripeptides is mediated by a single Na^+-independent, H^+-dependent transporter, named Peptide transporter 1 (PepT1) (Verri *et al.*, 2010). Following this apical influx, di- and tripeptides are hydrolysed in the enterocyte cytosol and the resulting AA cross the basolateral membrane using AA transporting systems. On the other hand, AA absorption through the BBM occurs either by Na+-independent transport, namely passive diffusion and facilitated transport, or by the Na+-dependent active transport (Collie and Ferraris, 1995). Both facilitated and active transports are mediated by proteins that recognise, bind and transport these molecules from the extracellular medium into the cell. Such transport proteins seems to be fairly conserved among vertebrates, although substrate specificity between fish and mammals differs. Efflux of nutrients from enterocytes to blood, across the basolateral membrane, is also similar to that found in mammals and, as with the BBM transporters, the rate of basolateral transport is smaller than in mammals, which is partly explained by the lower densities of transporters and smaller amounts of absorptive tissue in fish (Ferraris and Ahearn, 1984; Collie and Ferraris, 1995; Krogdahl *et al.*, 2005).

In fish, BBM AA absorption occurs predominantly by active transport, with transport proteins being energized by Na^+/K^+-ATPase (Jürss and Bastrop, 1995; Pan *et al.*, 2004; Krogdahl *et al.*, 2005). In contrast to mammals, fish AA transporters are present along the entire intestine, although the rates of nutrient uptake vary along the length of the intestine (Buddington and Diamond, 1987; Buddington *et al.*, 1997; Santigosa *et al.*,

2011). Some differences also exist between fish species, especially regarding sugar uptake, which is lower in carnivorous than in herbivorous and omnivorous fish, while AA uptake is similar between fish with distinct feeding regimes (Buddington *et al.*, 1997).

The intestinal AA transport systems that have been described for fish are complex and present overlapping specificity, as it is also the case in mammals (Collie and Ferraris, 1995). In fish, evidences exist of at least four different Na^+-dependent AA transporters specific for acidic, neutral, basic, and imino acids (Cassano *et al.*, 1990). Since several AA share the same transporter, but present distinct affinities to each specific transporter, the relative concentration of an AA in the lumen may affect the rate of absorption of another AA. This is the case of the neutral AA glutamine (Gln), which transport is inhibited by Alanine (Ala), Serine (Ser), Cysteine (Cys) and Leucine (Leu) (Fan *et al.*, 1998; Berge *et al.*, 2004; Pan *et al.*, 2004). Likewise, in European sea bass (*Dicentrarchus labrax*) cross-inhibition experiments revealed that glycine (Gly), alanine, and Met share the same Na+-dependent transporter, and mutual inhibition of alanine and Met transport by Gly occurs (Balocco *et al.*, 1993). Such interactions are especially relevant nowadays, given the increasing replacement of dietary FM by alternative protein sources with distinct AA profiles, which may potentially compromise the absorption of some AA, thus reducing their availability to the animal (Berge *et al.*, 2004).

Moreover, studies in mammals revealed that, due to transporters specificity for several AA, the majority of AA are not effective inducers of their main transporters. For instance, arginine (Arg), a basic AA, is the second-best inducer of acidic AA transporters, while aspartate (Asp), an acidic AA, is the best inducer of basic AA transporters (Stein *et al.*, 1987; Ferraris and Diamond, 1989; Salloum *et al.*, 1990; Wu, 2013a). However, in rat, high doses of dietary Gln supplementation (30% total dietary nitrogen provided by Gln) were shown to increase up to 75% the absorption capacity of the small intestine brush border membrane vesicles (BBMV) (Salloum *et al.*, 1990). Additionally, herbivorous and omnivorous fish were previously shown to be able to adapt the BBM glucose transport according to changes in dietary carbohydrate levels, through alteration of membrane transporters density or, to a less extent, by altering tissue mass (Collie and Ferraris, 1995; Krogdahl *et al.*, 2005). These results reveal fish capacity to adapt BBM to alterations in dietary composition, but further studies are needed to fully evaluate the potential of specific AA as modulators of BBM AA transport.

The BBMV is a technique that has been used to study intestinal nutrient transport in several fish species (Drai *et al.*, 1990; Ahearn *et al.*, 1992; Maffia *et al.*, 1996; Boge *et al.*, 2002; Sala-Rabanal *et al.*, 2004). Compared to other approaches that may also be used to determine uptake capacity, such as the everted sleeve technique or *in vivo*

apparent absorption (Bakke-McKellep *et al.*, 2000; Refstie *et al.*, 2006), the BBMV technique has the advantage of allowing the study of uptake rates without substrate metabolization or further basolateral transport, while also minimising the diffusion phenomena (Schep *et al.*, 1997) by working at short incubation periods (5 s) and low substrate concentrations.

1.4. Fish AA metabolism

While dietary lipids can be stored as triglycerides and cholesterol in adipose tissue, muscles and liver, and dietary carbohydrates may be stored as glycogen in muscles and liver, dietary proteins cannot. Moreover body proteins are continuously being renewed through degradation and synthesis, a process known as protein turnover. Following protein degradation, up to 40-50% of the resultant AA are recycled, returning to the FAA-pool, which comprises a cell-specific intracellular pool and an extracellular pool, corresponding to blood AA, which allows for organs interactions. In the FAA-pool recycled AA join dietary AA and DAA synthesized by the fish and can then serve as precursors for the synthesis of new proteins, regulate key metabolic pathways, and supply all reactions in which AA are used, including energy generation through the citric acid cycle (Cowey and Luquet, 1983; Jürss and Bastrop, 1995; Halver and Hardy, 2002).

The metabolic machinery of intermediary metabolism in fish is similar to that of mammals, despite that carbohydrate and lipids are the preferred energy sources in mammals, while dietary protein and lipids are the major energy sources in the majority of fish species (Enes *et al.*, 2009). The main differences between intermediary metabolism of mammals and fish are related to the hormonal control of nutrients utilization and the final products of nitrogen excretion. Contrary to mammals, most fish do not have a functional urea cycle and directly excrete nitrogenous wastes into the water in the form of ammonia (NH_4^+), a process that requires little energy expenditure (Walton and Cowey, 1982; Dabrowski and Guderley, 2002; Halver and Hardy, 2002).

In fish, as in mammals, the liver is a major site of metabolism and synthesis, carbohydrates and lipids being the main responsible for fish metabolic homeostasis (Sargent, *et al*., 2002; Enes *et al.*, 2009). The liver is the most important organ governing AA homeostasis. In fact, it is the major organ responsible for DAA synthesis, has the highest protein synthesis rate and a high protein turnover rate, justifying its high catabolizing enzymes activities (Walton and Cowey, 1982; Fauconneau and Arnal, 1985; Jürss and Bastrop, 1995).

Following feed intake, absorbed AA reach the liver through the hepatic portal system and, depending on fish nutritional status and the dietary protein quality, AA are used for protein synthesis, for energy production via the citric acid cycle, or used for glucose (through gluconeogenesis) or fatty acid (through lipogenesis) synthesis. The first step in AA catabolism consists in removing the amino group of the AA. For a few AA this may occur through direct deamination by specific enzymes, with ammonia formation. However, the majority of AA follow a transamination pathway, in which transaminases (or aminotransferases) catalyse the reversible transfer of an AAs' amino group to an α-keto acid, generating a new AA. Following the removal of the amino group, the carbon chain (α-keto acid) enters the citric acid cycle at different points (Figure 2A) to generate energy or to be converted into energy-storage products, namely glycogen and lipids, through the gluconeogenic and lipogenic pathways, respectively (Cowey and Sargent, 1979; Cowey and Walton, 1989; Jobling, 1994; Jürss and Bastrop, 1995; Thillart and Van Raaij, 1995). Thus, the transamination pathway is frequently the first step in AA degradation and the last step in DAA biosynthesis (Figure 2A and B).

In teleosts over 70% of ammonia is produced from AA catabolism in the liver, mainly by transdeamination (Cowey and Walton, 1989; Jürss and Bastrop, 1995). In this pathway an AA is transaminated and eventually originates glutamate (Glu), which is then oxidatively deaminated by glutamate dehydrogenase (GDH; EC 1.4.1.2), to α-ketoglutarate and ammonia (Figure 3). Alanine aminotransferase (ALAT, EC 2.6.1.2) and aspartate aminotransferase (ASAT, EC 2.6.1.1), are responsible for the transamination steps, converting Ala and Asp to Glu, respectively, being considered, along with GDH, the most relevant enzymes in AA catabolism (Engelking, 2015).

ALAT is present in cytosol and ASAT is present in both cytosol and mitochondria, and GDH is located in the mitochondrial matrix, making transdeamination a mitochondrial process. Moreover, being an allosteric enzyme, which is inhibited by ATP and GTP, and activated by ADP, GDH represents a key enzyme of control for AA catabolism (Cowey and Walton, 1989; Jürss and Bastrop, 1995; Engelking, 2015).

Hepatic AA catabolizing enzymes activities have been used as indicators of dietary AA metabolic utilization, with the dietary replacement of protein-bound AA by crystalline-AA or dietary AA imbalance being reported to affect ALAT, ASAT and GDH specific activities (Moyano *et al.*, 1991; Gomez-Requeni *et al.*, 2003; Peres and Oliva-Teles, 2005, 2006, 2007; Zhou *et al.*, 2011; Bañuelos-Vargas *et al.*, 2014). However, in fish, the effects of dietary AA profile on these enzymes activities is relatively contradictory, with several studies reporting an absence of regulation of some of these enzymes despite the changes in dietary AA profiles (Moyano *et al.*, 1991; Gomez-Requeni *et al.*, 2003; Peres

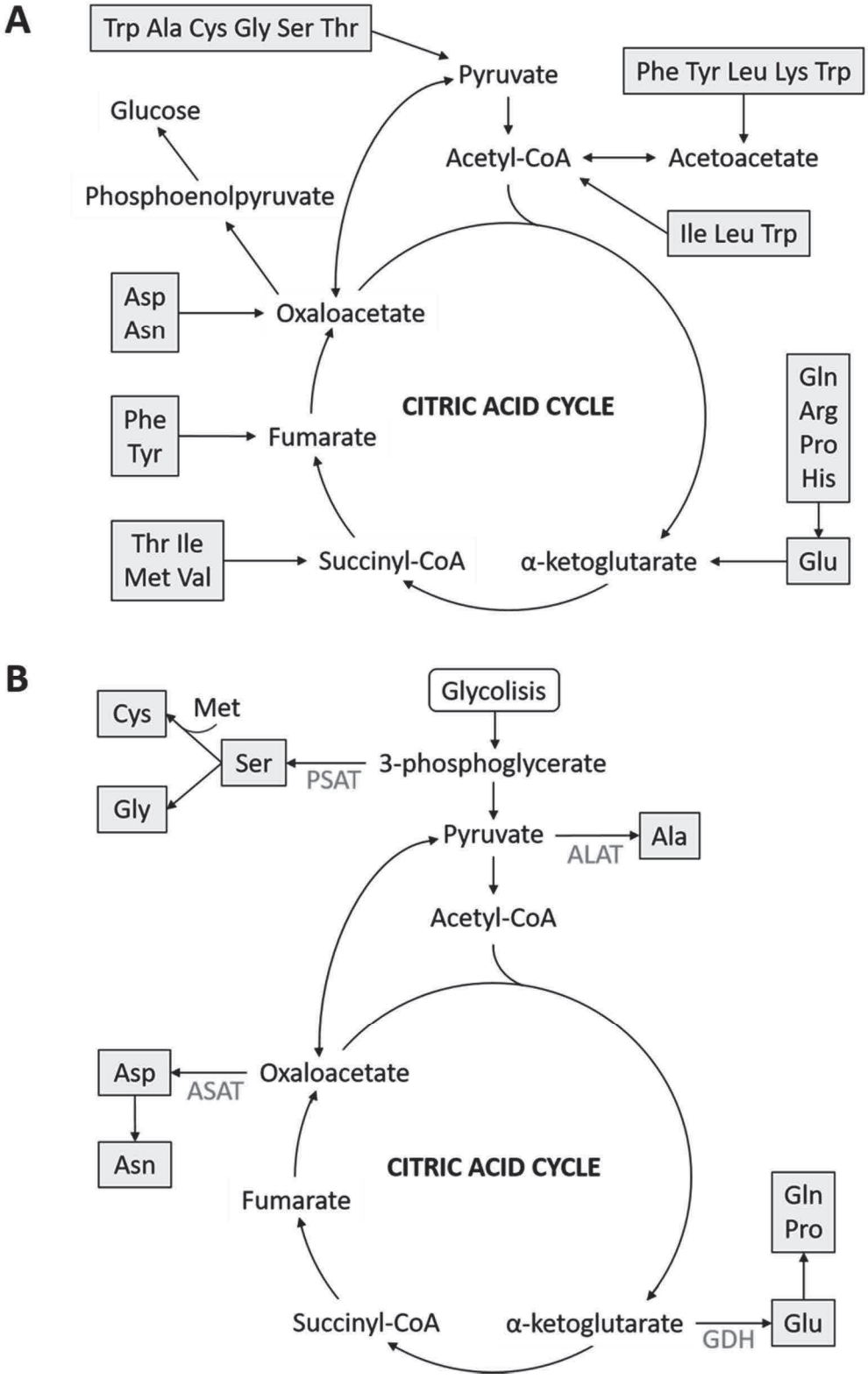

Figure 2. Interconversion of citric acid cycle intermediates and AA. A - Citric acid cycle intermediates formed following AA catabolism. B - DAA biosynthesis from citric acid cycle intermediates. Alanine (Ala); Tryptophan (Trp); Cysteine (Cys); Glycine (Gly); Threonine (Thr); Serine (Ser); Leucine (Leu); Tyrosine (Tyr); Phenylalanine (Phe); Lysine (Lys); Isoleucine (Ile); Histidine (His); Proline (Pro); Glutamine (Gln); Arginine (Arg); Glutamate (Glu); Valine (Val); Asparagine (Asn) Aspartate (Asp); Methionine (Met); Phosphoserine aminotransferase (PSA); Alanine aminotransferase (ALAT); Aspartate aminotransferase (ASAT); Glutamate dehydrogenase (GDH). Adapted from Engelking (2015).

and Oliva-Teles, 2006, 2007) or even increased ammonia excretion (Rolland *et al.*, 2016). In fact, Cowey (1995) considered fish hepatic AA catabolizing enzymes to be non-adaptive, while Moyano *et al.* (1991) reported GDH and transaminase enzymes activities to be highly susceptibility to different nutritional factors.

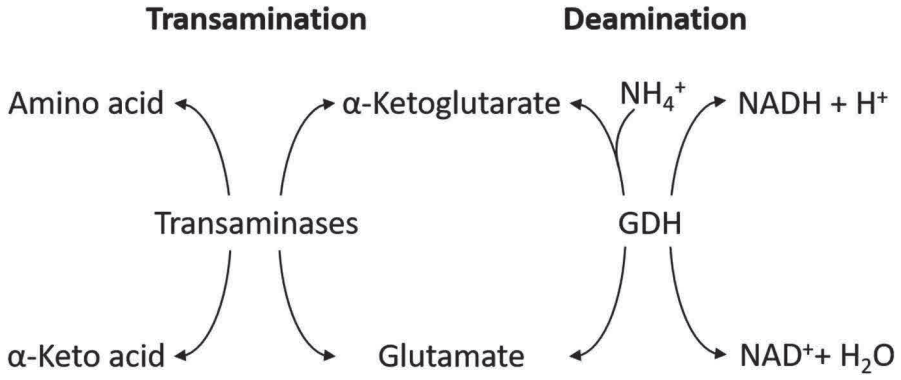

Figure 3. Transamination and deamination reactions of amino acids transdeamination pathway. Glutamate dehydrogenase (GDH); NH_4^+ - ammonium cation; Nicotinamida adenina dinucleótido hidreto (NADH); Nicotinamide adenine dinucleotide (NAD^+). Adapted from Jobling (1994).

Unlike the liver, the intestine is able to absorb AA from digested feed, through the BBM, and from the blood flow, through the basolateral membrane (Reshkin *et al.*, 1988; Collie and Ferraris, 1995; Jürss and Bastrop, 1995). Due to the constant renewal of the intestinal epithelium and enterocytes rapid protein turnover rates, the intestine requires a constant supply of AA, especially Gln. Still, similarly to liver, ALAT, ASAT and GDH are also the main AA catabolic enzymes in the intestine. While ALAT and ASAT present a lower activity in the intestine than in the liver, GDH activity, in percentage of body weight, was shown to be higher in the intestine than in the liver (Jürss and Bastrop, 1995). This fact and the reported basolateral uptake of glutamic acid (the hydrolysed form of Gln) by eel enterocytes are indicative that, as in mammals, Gln must be absorbed from the circulation in the post-absorptive phase to be extensively oxidised, in order to supply the citrate cycle with α-ketoglutarate for energy generation (Jürss and Bastrop, 1995).

1.5. Oxidative stress

Reactive oxygen species (ROS) are naturally generated in biological reactions involving oxygen, such as leukocytes respiratory burst, oxidative enzymatic activities, as for instance xanthine oxidase, tryptophan dioxygenase, diamine oxidase or prostaglandin synthase, and the mitochondrial electron-transfer chain. This last process is responsible for generating a proton gradient that is used for ATP synthesis and is one of the main endogenous source of ROS (Wood, 2000; Halliwell and Gutteridge, 2015; Ozcan and Ogun, 2015). Such process is responsible for the generation of superoxide radical ($O_2^{-}\cdot$), which may be reduced to hydrogen peroxide (H_2O_2). Through Feton reaction or Heber-Weiss reaction the H_2O_2 produced may then generate hydroxyl radical (·OH), an extremely reactive oxygen species able to damage virtually all types of macromolecules, being a major cause of cell injury (Figure 4). Singlet oxygen ($^1O_2$) is another active oxygen species that can be generated by biological systems (Storey, 1996; Sargent, *et al*., 2002; Ozcan and Ogun, 2015).

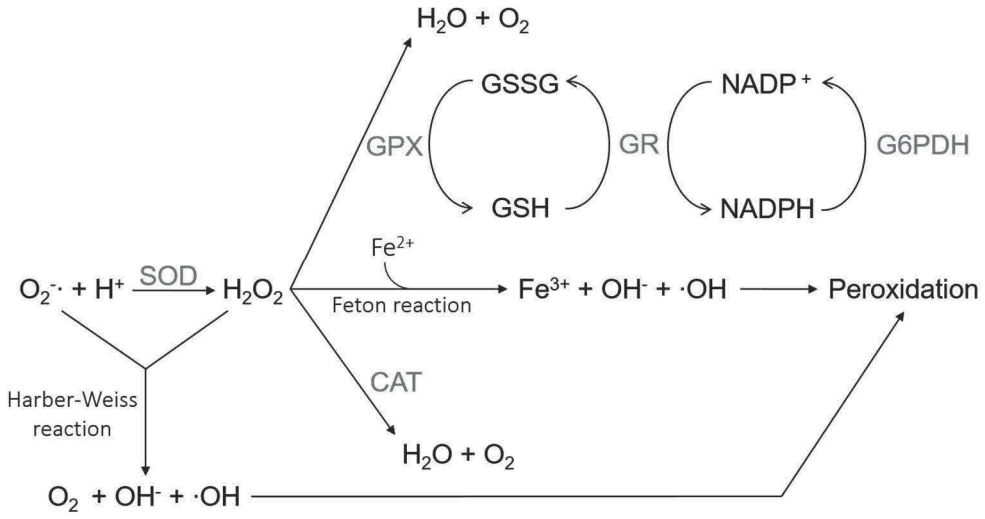

Figure 4. Summary of the pathways involved in reactive oxygen species generation and of some antioxidant enzymes actions. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PD) reduced glutathione (GSH), oxidized glutathione (GSSG). Adapted from Storey (1996).

Moreover, nitric oxide (NO) and several reactive nitrogen species (RNS) derived from its reaction with superoxide radical, namely, nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^-$), are also naturally generated in biological reactions involving oxygen. Both RNS and ROS have important roles in cellular signalling, in the regulation of vasodilatation and in the effective mounting and function of the immune response, being produced by

phagocytic cells and being responsible for killing engulfed microorganisms (Storey, 1996; Ellis, 2001; Ozcan and Ogun, 2015).

Under normal physiological conditions, ROS and RNS production and elimination are kept in balance. However if such a balance is lost and the reactive species reach high concentrations, these molecules readily react with proteins, lipids, and DNA, causing significant damage to cell's membrane integrity, impairing cellular metabolism or inducing apoptosis (Storey, 1996; Sargent *et al.*, 2002; Halliwell and Gutteridge, 2015; Ozcan and Ogun, 2015). This condition is known as oxidative stress and may lead to weakening of fish health status (Tocher *et al.*, 2002; Morales *et al.*, 2004).

Polyunsaturated fatty acids (PUFA), both isolated and incorporated into phospholipids and triglycerides, are extremely susceptible to ROS oxidation, producing lipid peroxides, a process known as lipid peroxidation. Since fish are rich in PUFA they are particularly vulnerable to lipid peroxidation, which has been shown capable of increasing membrane permeability and inactivating membrane-bound enzymes, resulting in disorders such as haemolysis, anaemia, jaundice, liver degeneration and skeletal alterations. Lipid peroxidation gains further importance given that it occurs through chain-reaction, whereby a single radical species, after extracting a hydrogen atom from a fatty acid, originates an unstable lipid radical that most likely will originate a peroxyl radical, propagating the reaction (Halliwell and Chirico, 1993; Sargent *et al.*, 2002; Mourente *et al.*, 2007). As a final product of lipid peroxidation, malonyldialdehyde is commonly used as marker for oxidative stress (Halliwell and Chirico, 1993).

To avoid the development of oxidative stress, aerobic animals present an antioxidant system that comprises both enzymatic and non-enzymatic antioxidants, and has a preventive action, delaying or destructing the oxidation substrates. Additionally, aerobic animals also present a secondary defence mechanisms (namely lipolytic, proteolytic and DNA repair enzymes, such as polymerases and other enzymes), responsible for repairing and removing damaged cellular components (Halliwell and Gutteridge, 2015).

Fish endogenously produce several non-enzymatic antioxidants. However, the biosynthesis of such antioxidants is frequently dependent on the dietary supply of several compounds and AA. For instance, nicotinic acid is required for nicotinamide adenine dinucleotide hydrate (NADH) / nicotinamide adenine dinucleotide phosphate (NADPH) synthesis and Cys, Glu, Gly are indispensable for glutathione (GSH) synthesis. In addition, fish are also supplied through the diet with other nutrients with important antioxidant roles. These include the AA taurine (Tau) and Met, vitamins C and E, minerals (selenium), or bioactive compounds of plant origin (phenolic and polyphenolic

compounds) with significant roles in the antioxidant defence (Storey, 1996; Martinez-Alvarez *et al.*, 2005; Perez-Jimenez *et al.*, 2012; Bañuelos-Vargas *et al.*, 2014).

Fish enzymatic antioxidant defence includes: cytosolic and mitochondrial superoxide dismutase (SODs; EC 1.15.1.1); peroxisomal and mitochondrial catalase (CAT; EC 1.11.1.6); and cytosolic and mitochondrial glutathione peroxidase (GPX; EC 1.11.1.19) and glutathione reductase (GR; EC 1.8.1.7). Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1 .1.49) does not have a direct antioxidant action, however, its role in the generation of NADPH is crucial for the normal functioning of CAT, GPX and GR (Mourente *et al.*, 2002; Morales *et al.*, 2004).

Antioxidant enzymes work together as the front line against oxygen radicals, by preventing the initialization of chain reactions triggered by superoxide radical. SOD is the first enzyme to respond to oxygen radicals, catalysing superoxide radical dismutation to molecular oxygen and hydrogen peroxide. Hydrogen peroxide is then converted by CAT to molecular oxygen and water, or reduced to water by GPX, through a GSH dependent reaction. GR is responsible for regenerating reduced glutathione (GSH) from the so formed oxidized glutathione (GSSG) at the expense of NADPH (Figure 4) (Storey, 1996; Mourente *et al.*, 2002; Morales *et al.*, 2004; Perez-Jimenez *et al.*, 2009; Halliwell and Gutteridge, 2015).

1.6. Nutrition-based health approach and functional AA

Until last decade AA were traditionally classified as IAA, DAA, and CIAA, few attention being given to their functional roles. Besides being required for protein synthesis, AA regulate cell signalling, gene expression, antioxidant responses, and immunity. AA are also used as precursors for the synthesis of small peptides (e.g., GSH), non-peptide hormones (e.g., epinephrine, norepinephrine, and thyroxine) and several low-molecular-weight nitrogenous metabolites (e.g., ammonia, carnitine, creatine, dopamine, NO, nucleotides, polyamines, and thyroxine) of major biological importance. The growing body of research in this area has led to the new concept of functional AA (Wu, 2010, 2013b), that takes into consideration the metabolic needs for dietary AA beyond tissue proteins synthesis and includes IAA, DAA and CIAA. Functional AA are those AA that participate in and regulate key metabolic pathways and that have potential to improve several aspects of animals' health, development, growth, or even survival (Wu, 2010; Kiron, 2012; Wu, 2014; Wu *et al.*, 2014).

1.6.1. Glutamine

Gln is the most prevalent AA in body fluids and muscle. Gln is the main systemic nitrogen carrier, having a major role in ammonia detoxification. Gln turnover rate exceeds that of other AA and increases under rapid growth or malnutrition or disease conditions. The increased awareness of Gln functional proprieties in health maintenance, immune function, and gastrointestinal function and integrity justified that Gln is now considered a CIAA (Li *et al.*, 2009; Wu, 2010; Wu, 2014; Wang *et al.*, 2016a).

Gln can be synthesized from Glu and ammonia by ATP-dependent glutamine synthetase (GSase; E.C. 6.3.1.2), or be degraded by phosphate-activated glutaminase (GlNase; EC 3.5.1.2) to Glu and ammonia (Xi *et al.*, 2011; Watford, 2015). Additionally, Glu can be synthesized from or catabolized to α-ketoglutarate through the reversible action of GDH and transaminase enzymes. This explains why the bulk of Gln hydrolysed by GlNase is either used as hepatic substrate for gluconeogenesis or for energy production through the citric acid cycle.

Gln is the major energy substrate of rapidly proliferating cells, as leucocytes, intestinal mucosal cells and enterocytes (Li *et al.*, 2009; Wu *et al.*, 2011; Wang *et al.*, 2016a). Gln and glutamic acid are also among the most important AA in fish liver oxidation and gluconeogenesis processes (Jürss and Bastrop, 1995). Moreover, Gln and Glu comprise a major proportion of the AA body pool, both in free form and incorporated into protein, and since their dietary supply is almost completely catabolized in the intestine, nearly all Gln and Glu body pool has to be synthesized endogenously (Li *et al.*, 2009; Watford, 2015; Wang *et al.*, 2016a).

In mammals, besides its role in protein synthesis and as a key energy source for rapidly proliferating cells, Gln is the most versatile AA in cellular metabolism, intervening in several Gln-dependent regulatory and biosynthetic pathways indispensable for growth and normal cell development. These include purines, pyrimidine, amino sugars, and NO synthesis; gene expression regulation; and activation of the mammalian target of rapamycin (mTOR), responsible for stimulating anabolic functions such as protein synthesis, cell growth and differentiation. Moreover, increasing intracellular Gln concentration stimulates protein synthesis and inhibits proteolysis in muscle and enterocytes (Wu *et al.*, 2011; Xi *et al.*, 2011; Watford, 2015; Wang *et al.*, 2016a).

Intestinal Gln metabolism and its role on intestinal health are of particular interest since Gln, besides being extensively catabolised by intestinal cells, plays an essential role in maintaining intestinal integrity and function; inhibits expression of inflammatory

cytokines; up-regulates expression of antioxidant genes; prevents endotoxin or oxidant-induced apoptosis, and modulates intestinal oxidative status, due to its role as GSH precursor (Reeds *et al.*, 2000; Curi *et al.*, 2005; Brasse-Lagnel *et al.*, 2009; Wu *et al.*, 2011; Xi *et al.*, 2011; Wu, 2014; Wu *et al.*, 2014). Since intestinal Gln metabolism relies extensively on feeding, maintenance of normal intestinal physiology is critically dependent on dietary Gln provision. Indeed, Gln has been recognised as a functionally active AA to support maximum growth and performance in swine nutrition (Wu *et al.*, 2011; Xi *et al.*, 2011).

Although tissue-specific metabolism of Gln and Glu is not fully described in fish (Li *et al.*, 2009), evidence exist that dietary Gln supplementation also enhances weight gain and feed efficiency (Yan and Qiu-Zhou, 2006; Cheng *et al.*, 2011; Qiyou *et al.*, 2011; Cheng *et al.*, 2012; Han *et al.*, 2014; Caballero-Solares *et al.*, 2015; Hu *et al.*, 2015; Pereira *et al.*, 2017), intestine structure and function (Qiyou *et al.*, 2011), antioxidant defences, including GSH content (Chen *et al.*, 2009; Li *et al.*, 2009; Cheng *et al.*, 2011; Li *et al.*, 2013; Han *et al.*, 2014; Hu *et al.*, 2014; Hu *et al.*, 2015), serum lysozyme and complement activities (Lobley *et al.*, 2001; Cheng *et al.*, 2011; Zhu *et al.*, 2011; Cheng *et al.*, 2012; Hu *et al.*, 2015; Zhang *et al.*, 2017), promotes enterocytes and leucocytes growth (Jiang *et al.*, 2009; Pohlenz *et al.*, 2012b), improves the repair activity in fish enterocytes challenged with H_2O_2 (Hu *et al.*, 2014), modulates cytokine gene expression and TOR kinase activity (Hu *et al.*, 2015).

1.6.2. Arginine

Arg is an IAA required for protein synthesis and somatic growth and serves important regulatory functions in nutrient metabolism and immune response. The *de novo* Arg synthesis is limited or even inexistent in the large majority of fish species, due to the lack of carbamoyl phosphate synthetase III (CPS III) activity, which is a pre-requisite for a functional ornithine-urea cycle (Anderson and Walsh, 1995; Todgham *et al.*, 2001; Gouillou-Coustans *et al.*, 2002; Tulli *et al.*, 2007; Ip and Chew, 2010). Nonetheless, rainbow trout (*Oncorhynchus mykiss*) (Rodehutscord *et al.*, 1995; Fournier *et al.*, 2002) and channel catfish (*Ictalurus punctatus*) (Buentello and Gatlin, 2000) juveniles were previously reported to show CPS III activity in the liver, although a functional urea cycle was not detected. On the other hand, elasmobranchs and ureogenic teleosts can at least partially meet their Arg requirements by synthesising Arg from citrulline (which may be

originated from Gln) through a fully functional ornithine-urea cycle (Mommsen *et al.*, 2001; Li *et al.*, 2009; Ip and Chew, 2010).

However, the activity of the key enzyme CPS III appears to be highly variable in adult teleosts and is still not completely elucidated in different species (Anderson, 2001). Such interspecific differences in the ability to endogenously synthesise Arg is pointed as the probable cause for the interspecific variability in Arg requirement estimates among fish species (NRC, 2011). Although Ball *et al.* (2007) defended that, with the exception of Pacific salmon (*Oncorhynchus* spp.), Arg requirements of teleosts should be around 1.2-1.5% of the diet, or 3.7-3.9% of dietary protein, it was previously evidenced that marine fish seem to have higher Arg requirements than salmonids (Coutinho *et al.*, 2012).

In fish, as in higher vertebrates, Arg deamination by arginase (argininolysis) originates urea and ornithine, the pivotal precursor for polyamines synthesis, which have a critical role in cell growth, proliferation and differentiation (Mommsen *et al.*, 2001; Walsh and Mommsen, 2001; Buentello *et al.*, 2007; Andersen *et al.*, 2013; Wu, 2013b). Moreover, Arg is also involved in the synthesis of proline and creatine and it is the sole precursor of NO production by macrophages during immune response (Buentello and Gatlin, 1999; Morris, 2007; Wu *et al.*, 2009; Yue *et al.*, 2015).

Arg also increases the release of pancreatic hormones involved in fish growth, such as glucagon (Vega-Rubín de Celis *et al.*, 2004), somatostatin (Eilertson and Sheridan, 1995), growth hormone (GH) and insulin-like growth factor 1 (IGF-1) (Plisetskaya *et al.*, 1991; Lall *et al.*, 1994; Baños *et al.*, 1999; Mommsen *et al.*, 2001; Andoh, 2007; Sink and Lochmann, 2007; Liang *et al.*, 2016). Moreover, Arg is also a more potent insulinotropic than glucose in fish, thus contributing to the improvement of glucose utilization for energy purposes and to the increment of AA uptake by tissues, through an insulin-mediated mechanism (Tulli *et al.*, 2007). In addition, Arg was shown to regulate fish mTOR signalling pathway (Tu *et al.*, 2015; Liang *et al.*, 2016), and plasma free Arg levels were reported to decrease in fish under stressful conditions (Aragão *et al.*, 2008; Costas *et al.*, 2008). Optimum dietary Arg levels were also shown to increase muscle GSH content and the activities of antioxidant enzymes, suggesting that Arg is able to modulate fish antioxidant response, although the pathways involved are still unclear (Wang *et al.*, 2015).

The strong endocrine modulatory capacity of Arg seem to explain the enhanced growth and feed efficiency (Tulli *et al.*, 2007; Oehme *et al.*, 2010; Cheng *et al.*, 2011; Cheng *et al.*, 2012; Zhou *et al.*, 2012a; Andersen *et al.*, 2013; Pohlenz *et al.*, 2013; Pohlenz *et al.*, 2014; Neu *et al.*, 2016), enhanced macrophages production, respiratory burst, lysozyme

activities and resistance to bacterial challenge (Buentello and Gatlin, 2001; Yue *et al.*, 2015; Zhang *et al.*, 2017) observed in fish fed dietary Arg surplus.

However, in a number of studies fish growth performance and feed efficiency were impaired due to a toxic effect of excess dietary Arg (Kaushik and Fauconneau, 1984; Kaushik *et al.*, 1988; Santiago and Lovell, 1988; Ngamsnae *et al.*, 1999; Fournier *et al.*, 2003; Singh and Khan, 2007; Abidi and Khan, 2009; Zhou *et al.*, 2011; Zhou *et al.*, 2012b; Ren *et al.*, 2013; Van Nguyen *et al.*, 2014; Lin *et al.*, 2015), which could be a consequence of the extra energy expenditure toward Arg deamination (Zhou *et al.*, 2012a; Ren *et al.*, 2013; Zhou *et al.*, 2015). An Arg-Lys antagonism that limits Lys uptake might also explain this negative effect of excess dietary Arg (Kaushik *et al.*, 1988; Berge *et al.*, 1999; Ren *et al.*, 2013; Van Nguyen *et al.*, 2014), but such antagonism is yet to be demonstrated in fish (Alam *et al.*, 2002; NRC, 2011; Andersen *et al.*, 2014).

1.6.3. Methionine

Met is generally the first limiting AA in many plant protein sources, including soybean meal, rapeseed meal, lupin meal, pea protein concentrate and soy protein concentrate (ARRAINA, 2015; Oliva-Teles *et al.*, 2015), which justifies its availability as feed grade (Li *et al.*, 2009; Pan *et al.*, 2016).

Fish, as other animals, have both Met and total sulphur AA (TSAA; Met + Cys) requirements (Wilson, 2002) and since Met is an irreversible precursor of Cys, TSAA requirements might be met by Met alone or by a combination of Met and Cys. Although Cys production through Met transsulfuration pathway is 100% efficient in sulphur molar basis, the different molecular weight of these two AA (149.2 g/mol for Met and 121.2 g/mol for Cys) imply that 149.2 mg of Met yield only 121.2 mg of Cys, resulting in a 19% loss of Met in weight terms (Baker, 2006). This, associated with the traditional estimation of Met and TSAA requirements using diets with low levels or even devoid of Cys, explain the 39% to 60% reduction of dietary Met requirements observed in several fish species when dietary Met:Cys ratio is decreased under optimal dietary TSAA level (Harding *et al.*, 1977; Griffin *et al.*, 1994; Goff and Gatlin, 2004; Nguyen and Davis, 2009; Tulli *et al.*, 2010; Abidi and Khan, 2011; Farhat and Khan, 2014; Klatt *et al.*, 2016; Poppi *et al.*, 2017).

Cys is key precursor of GSH and Tau, and therefore these two potent antioxidants can be indirectly produced from Met (Keembiyehetty and Gatlin, 1995; Wu *et al.*, 2004; Li *et al.*, 2007; Métayer *et al.*, 2008), although the capability to synthetize Tau in fish seems

to be species specific (Yokoyama *et al.*, 2001; Salze and Davis, 2015). Met is also precursor of S-Adenosylmethionine (SAM), which is an important methyl group donor for spermidine and L-carnitine synthesis (Schuhmacher and Gropp, 1998; Métayer *et al.*, 2008; Li *et al.*, 2009). In eukaryotic organisms, Met is also known to act as an initiating factor in protein synthesis and to inhibit proteolysis (Métayer *et al.*, 2008; Poppi *et al.*, 2017). Additionally, evidences exist that dietary TSAA level influences ascorbic acid metabolism, while dietary ascorbic acid supplementation was shown to reduce the synthesis of GSH and Cys from Met (Li *et al.*, 2007; Métayer *et al.*, 2008; Li *et al.*, 2009).

Due to its role as methyl group donor for DNA methylation, Met influences the expression of several genes related to the production of antioxidant enzymes (Seneviratne *et al.*, 1999; Métayer *et al.*, 2008). Moreover, the association of protein-exposed Met residues with the enzyme methionine sulfoxide reductase forms the antioxidant “methionine sulfoxide reductase” (MSR) system. In this system, Met residues are readily oxidized by several ROS originating methionine sulfoxide, which is then reduced back to Met by methionine sulfoxide reductase (Levine *et al.*, 2000). Through this system, exposed Met residues protect other functionally essential residues from ROS, thus limiting the oxidative damage and the accumulation of injured proteins in the cells (Levine *et al.*, 2000; Métayer *et al.*, 2008).

Accumulated evidences also suggest that the signalling role of Met is well conserved between lower and higher vertebrates (Belghit *et al.*, 2014), with Met exerting greater regulatory effect on gene expression in fish than other AA, such as Lys. In fish, Met was also shown to directly or indirectly affect the hepatic expression of genes involved in the GH/IGF system and in the two major AA signalling pathways, TOR and the general control nonderepressible 2 (GCN2), and to regulate the expression of hepatic intermediary metabolism related genes, which explains Met involvement in protein turnover and growth regulation (Lansard *et al.*, 2011; Belghit *et al.*, 2014; Rolland *et al.*, 2015; Rolland *et al.*, 2016; Skiba-Cassy *et al.*, 2016; Wang *et al.*, 2016b).

In fish, dietary methionine deficiency was reported to suppress IGF-1 gene expression (Wang *et al.*, 2016b), reduce feed intake, feed efficiency, and weight gain (Mambrini *et al.*, 1999; Zhou *et al.*, 2006; Tulli *et al.*, 2010; Nwanna *et al.*, 2012; He *et al.*, 2013; Belghit *et al.*, 2014; Figueiredo-Silva *et al.*, 2015; Rolland *et al.*, 2015; Poppi *et al.*, 2017), reduce muscle protein deposition (Belghit *et al.*, 2014) and total GSH (Keembiyehetty and Gatlin, 1995); increase protein degradation (Belghit *et al.*, 2014) and lipid peroxidation (Li *et al.*, 2009; Espe *et al.*, 2010); and to cause bilateral cataracts (Cowey *et al.*, 1992; Simmons *et al.*, 1999) or even mortality (Li *et al.*, 2009).

On the opposite side, dietary Met supplementation (or the more affordable Met hydroxyl analogue, MHA), above requirements was reported to improve fish immune status, by increasing the peripheral leucocyte response, serum lysozyme and immunoglobulin M (IgM) levels, complement activity, and plasma peroxidase and bactericidal activities (Kuang *et al.*, 2012; Machado *et al.*, 2015; Pan *et al.*, 2016). Optimum dietary MHA levels was shown to down-regulate proinflammatory cytokines and to up-regulate anti-inflammatory cytokines mRNA levels and attenuated inflammation-induced oxidative damage, by increasing antioxidant enzymes activities and GSH content of immune organs (Kuang *et al.*, 2012; Pan *et al.*, 2016).

1.6.4. Taurine

Tau is a β-AA, not an α-AA, though it is usually considered together with AA. Tau is not incorporated into proteins, but is one of the most abundant FAA in animal tissues (Wang *et al.*, 2014; Salze and Davis, 2015). In fish, as in mammals, Tau can be synthesised from Cys through several enzymatic reactions, where cysteine dioxygenase (CDO) is the vital enzyme in regulating Cys concentration and cysteine sulfinic acid decarboxylase (CSD) is the rate-limiting step (Jacobsen and Smith, 1968; Wang *et al.*, 2014). Marine fish seem to present limited or no capacity to synthesize Tau (Yokoyama *et al.*, 2001; Park *et al.*, 2002; Takagi *et al.*, 2005; Wang *et al.*, 2014), mainly due to absence or low activity of CSD (Jacobsen and Smith, 1968). Thus, Tau is now considered a CIAA (Wang *et al.*, 2014; Salze and Davis, 2015) or even an IAA in some species (Goto *et al.*, 2001; Kim *et al.*, 2005; Qi *et al.*, 2012; Lim *et al.*, 2013).

Although Tau is abundant in FM, it is almost absent in PF (Spitze *et al.*, 2003; Li *et al.*, 2009; Wang *et al.*, 2014; Lall and Dumas, 2015), which implies that plant-based diets for some marine fish need to be supplemented with Tau (Wang *et al.*, 2014; Salze and Davis, 2015). In several marine fish species, dietary Tau supplementation was shown to enhance performance of fish fed low FM-diets (Park *et al.*, 2002; Kim *et al.*, 2005; Matsunari *et al.*, 2005; Takagi *et al.*, 2006; Lunger *et al.*, 2007; Chatzifotis *et al.*, 2008; Enterria *et al.*, 2011; Lim *et al.*, 2013; Jirsa *et al.*, 2014; Khaoian *et al.*, 2014; Hien *et al.*, 2015; Wu *et al.*, 2015; Koven *et al.*, 2016) or all-plant diets (Takagi *et al.*, 2008; Takagi *et al.*, 2010). Similar effects were also observed in freshwater carnivorous fish, such as yellow catfish (*Pelteobagrus fulvidraco*) (Li *et al.*, 2016) and rainbow trout (Gaylord *et al.*, 2006), but not in herbivorous common carp (*Cyprinus carpio*) (Kim *et al.*, 2008) and omnivorous Nile tilapia (*Oreochromis niloticus*) (Koch *et al.*, 2016). In addition, Tau

supplementation to low FM-diet was reported to restore lipid peroxidation levels (Bañuelos-Vargas *et al.*, 2014; Li *et al.*, 2016), enhance the activity of key enzymes of the intermediary metabolism (Bañuelos-Vargas *et al.*, 2014) and plasma biological antioxidant potential (BAP) (Han *et al.*, 2014), and to increase the activity of several antioxidant enzymes (Bañuelos-Vargas *et al.*, 2014; Feidantsis *et al.*, 2014; Li *et al.*, 2016).

The positive effects of dietary Tau supplementation are thought to result from its involvement in a number of relevant physiological functions, including modulation of calcium levels, cell membrane stabilization, anti-oxidative, anti-inflammatory, and osmoregulatory functions, and on bile acid conjugation (Huxtable, 1992; NRC, 2011; Han *et al.*, 2014; Salze and Davis, 2015). In effect, dietary Tau deficiency is known to cause the nutritional disease known as “green liver syndrome”, in which there is overproduction of hemolytic biliverdin associated with decreased excretion of Tau-conjugated bile pigment (Sakai *et al.*, 1990; Goto *et al.*, 2001; Takagi *et al.*, 2005; Takagi *et al.*, 2006).

Tau is considered a potent antioxidant; however, its antioxidant role is yet to be fully understood. Since Tau is a weak ROS scavenger (Aruoma *et al.*, 1988), its antioxidant activity is more likely related to the prevention of mitochondrial oxidant production (Jong *et al.*, 2012; Salze and Davis, 2015). In fact, Tau regulates mitochondrial protein synthesis, thereby enhancing the activity of mitochondrial electron transport chain and avoiding electron donors accumulation, thus inhibiting the potential diversion of electrons to oxygen generating superoxide (Carneiro *et al.*, 2009; Schaffer *et al.*, 2009; Jong *et al.*, 2012).

1.7. Fish species studied

Gilthead sea bream (*Sparus aurata*, Linnaeus, 1758; Figure 5) and European sea bass (*Dicentrarchus labrax*, Linnaeus, 1758; Figure 6) are the two main marine species produced in southern Europe. Both species were traditionally produced under extensive or semi-intensive polyculture conditions in coastal lagoons and tidal reservoirs until the development of reliable mass production techniques during late 1960’s. Nowadays, these species are mainly farmed in large-scale intensive cages, with only a few semi-intensive farms remaining, mainly in Portugal and Spain (FAO, 2005a,b).

Between 2014 and 2015 marine fish production in the Mediterranean region increased 1.6% accompanying the modest growth of markets and consumers demand. In 2015,

the combined production of European sea bass and gilthead sea bream in southern Europe reached 300.000 t. with European sea bass production representing 49% of total marine species production. Gilthead sea bream production represented 45% of the total, which, when compared to 2012, corresponds to a reverse of position between the two species. Greece and Turkey are the main marine fish producers in southern Europe, followed by Spain and Italy. These four countries account for 94% of total production reported for European sea bass and gilthead sea bream, while Portugal, Cyprus, and France account for only 4% of these species production (FEAP, 2016).

1.7.1. Gilthead sea bream (*Sparus aurata*)

Gilthead sea bream (Figure 5) belongs to the Sparidae family, and is found in the Mediterranean and along the Eastern Atlantic Ocean, from British Isles to Cape Verde and around Canary Islands, being rare in the Black Sea. This euryhaline and eurythermic (5-32°C) species is benthopelagic, living in seagrass beds, rocky, and sandy bottoms from the surf zone up to 150m depth, although being more common at depths of about 30m. Gilthead sea bream is mainly carnivorous, having a trophic level of 3.3–3.5, and feeding on shellfish (mussels and oysters), crustaceous, fish and, sporadically, algae (Basurco *et al.*, 2011; FishBase, 2017a). It is a protandrous hermaphrodite species, beginning life as male and becoming female after reaching 33-40 cm with 2-3 years. Spawning takes place in the open sea, typically between October and December, with sequenced spawning during the whole period, with the juveniles returning to coastal waters in early spring (FAO, 2005a; Basurco *et al.*, 2011).

Figure 5. Gilthead seabream *(Sparus aurata).* Adapted from Fisheries-EU 2017.

1.7.1.1. Nutritional requirements

For gilthead sea bream juveniles, optimum dietary protein level was initially estimated to be 40% using semi-purified diets (Sabaut and Luquet, 1973). Later studies, using practical diets, reported dietary protein requirement of 45-46% in juveniles (Santinha *et al.*, 1996; Vergara *et al.*, 1996a) and as high as 55% for fry (Vergara *et al.*, 1996b).

Quantitative data on IAA requirements based on dose-response studies are only available for Arg, Lys, Met and Cys, and Trp (Table 2) (Luquet and Sabaut, 1974; Marcouli *et al.*, 2005; Marcouli *et al.*, 2006). Indirect estimations of IAA requirements were also made by the ideal protein method (Table 2), based on Lys requirement and whole-body IAA composition (Kaushik, 1998), and more recently by the AA deletion method (Table 2) (Peres and Oliva-Teles, 2009).

Table 2. Amino acid requirements (g/16 g N) of gilthead sea bream.

Methods	Dose reponse	Ideal protein	Amino acid deletion
Amino acid			
Arginine	<6	5.4	5.5
Histidine	-	1.7	1.89
Isoleucine	-	2.6	2.55
Leucine	-	4.5	4.75
Lysine	4.9-5	-	5.13
Methionine	-	-	2.6
Methionine + Cystine	2.8-4	2.4	-
Phenylalanine + Tyrosine	-	2.9	5.76
Threonine	-	2.8	2.98
Tryptophan	0.5-0.6	0.6	0.75
Valine	-	3.0	3.21

Adapted from: (Luquet and Sabaut, 1974; Kaushik, 1998; Marcouli *et al.*, 2005; Marcouli *et al.*, 2006; Peres and Oliva-Teles, 2009).

Optimum dietary lipid levels were established to be 15-16% by Vergara and Jauncey (1993) and Vergara *et al.* (1996a), but more recently this value was re-evaluated to 21-22% by Santinha *et al.* (1999) and Vergara *et al.* (1999). Even though dietary lipid levels up to 28% appeared to promote the best growth and to have protein sparing effect when low quality FM was used, the excess of lipids was pointed as the plausible cause of hepatocyte abnormalities observed in fish fed the highest dietary lipid levels (Vergara *et al.*, 1999). For that reason, the authors discourage the use of such high dietary lipid levels to avoid unwanted adiposity and adverse effects to fish health.

Regarding essential fatty acids requirements, Kalogeropoulos 1992 estimated that 1g gilthead sea bream fed a diet with 12% lipids (6% soybean oil+ 6% cod-liver oil) required 0.9% eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). More recently, (Benedito-Palos *et al.*, 2009) also reported that a dietary EPA + DHA level of 1% was sufficient to meet essential fatty acids needs of gilthead sea bream.

Although not having carbohydrate requirements, available data suggest that diets for gilthead sea bream juveniles could contain up to 20% of digestible carbohydrates, since higher dietary inclusion levels tend to depress fish growth and feed utilization (Peres *et al.*, 1999; Enes *et al.*, 2011).

Data on vitamin and mineral requirements of gilthead sea bream is scarce. Still, quantitative requirements exist for some vitamins: 63-83 mg/kg for niacin (Morris and Davies, 1995a), 0.5-5 mg/kg feed for thiamine (vitamin B_1) in juveniles (>60–200 g BW) (Morris and Davies, 1995b) and 2 mg/kg of dry diet for pyridoxine (vitamin B6) (Kissil *et al.*, 1981). Although vitamin C requirements were not estimated for gilthead sea bream, evidences show that vitamin C do not affect growth performance of fish fed a practical fishmeal-based diet (Henrique *et al.*, 1998). Regarding minerals, only phosphorus requirement was estimated, corresponding to 0.75% of the diet for juveniles (Pimentel Rodrigues and Oliva-Teles, 2001).

1.7.1.2. Fish meal replacement by plant feedstuffs

Several studies have been undertaken to evaluate the feasibility of FM replacement by single or mixtures of PF in compound diets for gilthead sea bream. Regarding the substitution of FM by single plant protein sources, it was shown that pea seed meal could replace up to 20% (Pereira and Oliva-Teles, 2002), lupin seed meal up to 30% (Pereira and Oliva-Teles, 2004), corn gluten meal up to 60% (Pereira and Oliva-Teles, 2003), rapeseed meal 20% (only one level tested) (Omnes *et al.*, 2015), rapeseed protein concentrate up to 40% (Kissil *et al.*, 1997), and soybean meal between 30 and 40% in juveniles and 50% for grow-out fish (Bonaldo *et al.*, 2008; Kokou *et al.*, 2012), without negatively affecting growth and feed efficiency.

By using mixtures of plant protein sources (corn gluten meal, wheat gluten, wheat, soy protein concentrate, extruded peas, rapeseed meal, extruded whole wheat) some authors manage to replace up to 75% (Gomez-Requeni *et al.*, 2004; De Francesco *et al.*, 2007) and 87% (Benedito-Palos *et al.*, 2016) of dietary FM with only slight or no reduction in weight gain. Using a mixture of PF (corn gluten, wheat gluten, extruded

peas, rapeseed meal and sweet white lupin) supplemented with IAA and keeping the IAA/DAA ratio of all diets close to 1.1, Sitja-Bobadilla *et al.* (2005) was also able to replace 75% of dietary FM without impairing fish growth performance, while Kissil and Lupatsch (2004) achieved a 100% FM replacement by a mixture of PF (corn gluten, wheat gluten, soy protein concentrate) supplemented with IAA (Met; Lys; Thr and Arg).

Although available data indicate that it is now feasible to include high plant protein levels in the diets for gilthead sea bream without compromising growth performance, such high FM replacement levels are still limitedly used, mainly due to economic constrains, resultant from the high prices of some PF (plant protein concentrates) when compared to FM, and also the high prices of some IAA (ex. Arg) (Kissil and Lupatsch, 2004).

It is also important to note that gilthead sea bream juveniles seem less tolerant than grow-out fish to the inclusion of plant protein sources in the diets (Martinez-Llorens *et al.*, 2007), which may be related to lower tolerance to ANF of juveniles than grow-out fish (Couto *et al.*, 2014). Indeed, saponins and phytosterols were shown to negatively affect several aspects of gilthead sea bream health, causing immune reactions at the distal intestinal level (the most sensitive region of the intestine to ANF) and some intestinal dysfunction. Even though such adverse effects are less pronounced in gilthead sea bream than in other species, such as salmonids (Krogdahl *et al.*, 2010), it must be assured that fish physiological function and intestinal health is not compromised by the inclusion of high plant protein levels in the diet (Couto *et al.*, 2014).

1.7.2. European sea bass (*Dicentrarchus labrax*)

European sea bass (Figure 6) is an euryhaline and eurythermic (2-32°C) fish of the Moronidae family (Hidalgo and Alliot, 1988; FAO, 2005b). This species is found in the coastal area from Norway to Morocco, Canary Islands and Senegal, as well as in the Mediterranean and Black Sea, inhabiting coastal inshore waters up to 100-150 m depth, and occurring in estuaries, brackish water lagoons and occasionally in rivers.

European sea bass is a carnivorous species with a trophic level of 3.8 (FishBase, 2017b) that typically feeds on small fish, invertebrates, prawns, crabs and cuttlefish. It is a dioecious (separate sex) species whose sexual maturity generally occurs at 2 to 4 years of age in the Mediterranean populations, while in the Atlantic populations sexual maturity occurs later, between 4-7 years of age (30 to 40 cm of length) for males and between 5-8 years of age (36-46 cm of length) for females. Spawning season occurs just once a year, mostly in winter for the Mediterranean population (December to March), extending

up to June for the Atlantic populations (FAO, 2005b; Pérez-Ruzafa and Marcos, 2015; Pozo *et al.*, 2015; FishBase, 2017b).

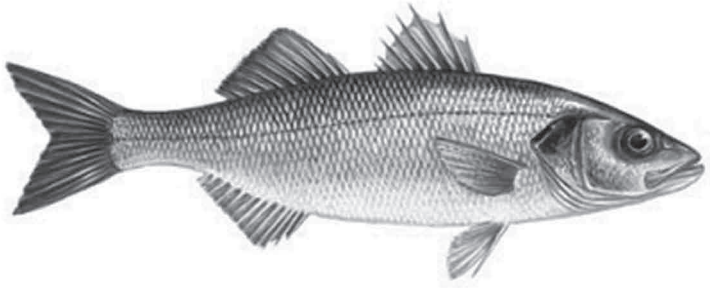

Figure 6. European sea bass (*Dicentrarchus labrax*). Adapted from Fisheries-EU, 2017.

1.7.2.1. Nutritional requirements

European sea bass protein requirement was first established as ranging from 52 to 60% (Alliot *et al.*, 1974; Metailler *et al.*, 1981). Later studies showed that dietary protein levels could be decreased to values between 48-54% (Hidalgo and Alliot, 1988; Ballestrazzi *et al.*, 1994; Peres and Oliva-Teles, 1999a), while other works were able to achieve optimum growth of this species with still lower dietary protein levels of 43-45% (Perez *et al.*, 1997; Dias *et al.*, 1998).

Estimates of IAA requirements based on dose-response studies are only available for Arg, phenylalanine (Phe), histidine (His), isoleucine (Ile), Leu, Lys, Met, Thr, Tyr and Val (Table 3) (Thebault *et al.*, 1985; Tibaldi and Lanari, 1991; Tibaldi *et al.*, 1993; Tibaldi *et al.*, 1994; Tibaldi and Tulli, 1999; Tulli *et al.*, 2010). IAA requirements (Table 3) were also indirectly estimated through the ideal protein method (Kaushik, 1998).

While some studies reported that increasing dietary lipid levels above 12% had no beneficial effects on European sea bass growth performance (Alliot *et al.*, 1974; Metailler *et al.*, 1981; Perez *et al.*, 1997; Peres and Oliva-Teles, 1999a), other studies indicated that fish growth performances was improved by increasing dietary lipids up to 18-19% (Dias *et al.*, 1998; Lanari *et al.*, 1999). It is however clear that increasing dietary lipid level up to 30% is not recommended for juveniles, as it appears to lead to a significant decrease in voluntary feed intake (Boujard *et al.*, 2004) and to compromise growth performance (Peres and Oliva-Teles, 1999b).

Table 3. Amino acid requirements (g/16 g N) of European sea bass.

Methods	Dose response	Ideal protein
Amino acid		
Arginine	3.9-4.6	4.6
Histidine	-	1.6
Isoleucine	-	2.6
Leucine	-	4.3
Lysine	4.8	-
Methionine	1.8-2.0	-
Methionine + Cystine	2.7-4.0	2.3
Phenylalanine + Tyrosine	-	2.6
Threonine	2.3-2.6	2.7
Tryptophan	0.5	0.6
Valine	-	2.9

Adapted from: (Thebault *et al.*, 1985; Tibaldi and Lanari, 1991; Tibaldi *et al.*, 1993; Tibaldi *et al.*, 1994; Kaushik, 1998; Tibaldi and Tulli, 1999; Tulli *et al.*, 2010)

Quantitative data on essential fatty acids requirements is scarce, with early estimates suggesting a requirement of about 1% of n-3 PUFA (Coutteau *et al.*, 1996) and a more recent work reporting a n-3 highly unsaturated fatty acids (HUFA) requirement of 0.7% for juveniles, with a DHA:EPA ratio of 1.5:1 (Skalli and Robin, 2004).

The incorporation of digestible carbohydrates in diets for European sea bass is recommended to be limited to 20%, as higher levels of starch (30%) appear to depress growth and feed utilization (Enes *et al.*, 2011).

Regarding vitamin and mineral requirements, Kaushik (1998) suggested that water-soluble vitamins allowances recommended for salmonids (NRC, 1993) can be used in practical diets for this species, but higher values should be used for purified diets. Phosphorous is the only mineral which requirement was studied, with an estimated requirement of circa 0.65% (Oliva-Teles and Pimentel-Rodrigues, 2004).

1.7.2.2. Fish meal replacement by plant feedstuffs

Pea seed meal, rice protein concentrate, and soybean meal were successfully used to replace 12%, 25%, and 25-30% of dietary FM protein, respectively, without negatively affecting European sea bass growth and feed utilization (Gouveia and Davies, 1998; Lanari and D'Agaro, 2005; Bonaldo *et al.*, 2008). If supplemented with the most limiting AA (Lys, Met, and Arg), wheat gluten can replace up to 70% of dietary FM protein (Messina *et al.*, 2013), while solvent-extracted (SE) soybean meal, enzyme-treated (ET) soybean meal and a mixture composed of 30% SE and 30% ET soybean meals may

replace 25%, 50%, and 60% of dietary FM protein, respectively, without hampering European sea bass performance (Tibaldi *et al.*, 2006).

Almost total replacement of dietary FM protein (95%) by plant protein mixture (corn gluten, wheat gluten, soybean meal, and rapeseed meal) with Lys supplementation was first achieved by Kaushik *et al.* (2004), without affecting fish growth, diet digestibility, or voluntary feed intake. Recently, Torrecillas *et al.* (2017) were also able to replace 90% dietary FM protein by a plant protein mixture (soy protein concentrate, corn gluten meal, wheat gluten, rapeseed meal, wheat) plus 5% blood meal, and Lys and Met supplementation, and with almost complete fish oil replacement by vegetable oil (3% fish oil). Moreover, the inclusion of 30% whole wheat, corn, rye and barley have also recently been shown not to cause histomorphological changes in the distal intestine of European sea bass (Couto *et al.*, 2017). Nowadays, commercial diets for European sea bass include up to 30-40% of dietary protein from soybean meal, corn or wheat gluten, a value that is expected to increase as knowledge progresses (Tacon and Hasan, 2011).

Although European sea bass seems to accept well PF as alternative protein sources, it is also known that ANF present in PF may also induce several side effects, such as: reduce the activity of BBM enzymes, causing inflammatory changes in the distal intestine (Couto *et al.*, 2015); up-regulate proinflammatory cytokines IL-1 β and TNFα gene expression; increase mucus production; and lead to changes in mucosal microbiota profile (Torrecillas *et al.*, 2017). Since the effects of ANF seem very much dependent of the dietary inclusion level (Couto *et al.*, 2015), further attention should be given to fish physiological function and intestinal health when considering the use of high PF inclusion levels in diets for European sea bass.

1.8. Objectives

Incorporating high levels of PF in aquafeeds without compromising performance and health of carnivorous fish still represents a challenge. Meanwhile, growing evidence reveals that functional AA seem to improve fish somatic growth, nutrient retention, gut health, and antioxidant defence.

Therefore, the overall objective of this thesis was to gain further knowledge on the potential benefits of supplementing practical/plant-based diets for the two major marine fish species produced in southern Europe aquaculture, gilthead sea bream and European sea bass, with four functional AA, namely glutamine (Gln), arginine (Arg), methionine (Met) and taurine (Tau). For that purpose, growth performance, feed utilization, body composition, intestinal nutrient absorption capacity, liver and intestine AA catabolism, and oxidative status, were evaluated.

Thus, Chapter 2 evaluates, in gilthead sea bream juveniles, the effects of three dietary Gln supplementation levels (0.5, 1, and 2%) on growth performance, whole-body and liver composition, intestinal Gln, Arg, and glucose absorption capacity, hepatic and intestinal AA catabolism, Gln metabolism and oxidative status.

Likewise, Chapter 3 assesses, also in gilthead sea bream, the effects of three Arg supplementation levels (0.5, 1, and 2%) on growth performance, whole-body and liver composition, intestinal Gln, Arg and glucose absorption capacity, hepatic and intestinal AA catabolism, and oxidative status.

Finally, Chapter 4 tested in European sea bass the effect of supplementing practical diets, with Met levels 12% below or 15% above the established requirement for the species, with 1% Tau. Both Met and Tau effects on European sea bass juveniles growth performance, whole-body composition, hepatic and intestinal oxidative status were evaluated.

Chapter 2

Dietary glutamine supplementation effects on amino acid metabolism, intestinal nutrient absorption capacity and antioxidant response of gilthead sea bream (*Sparus aurata*) juveniles

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Dietary glutamine supplementation effects on amino acid metabolism, intestinal nutrient absorption capacity and antioxidant response of gilthead sea bream (*Sparus aurata*) juveniles

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ABSTRACT

A study was undertaken to evaluate dietary glutamine supplementation effects on gilthead sea bream performance, intestinal nutrient absorption capacity, hepatic and intestinal glutamine metabolism and oxidative status. For that purpose gilthead sea bream juveniles (mean weight 13.0 g) were fed four isolipidic (18% lipid) and isonitrogenous (43% protein) diets supplemented with 0, 0.5, 1 and 2% glutamine for 6 weeks. Fish performance, body composition and intestinal nutrient absorption capacity were not affected by dietary glutamine levels. Hepatic and intestinal glutaminase (GlNase), glutamine synthetase (GSase), alanine aminotransferase, aspartate aminotransferase and glutamate dehydrogenase activities were also unaffected by dietary glutamine supplementation. In the intestine GlNase activity was higher and GSase/GlNase ratio was two-fold lower than in the liver, suggesting a higher use of glutamine for energy production by the intestine than by the liver. The liver showed higher catalase and glucose-6-phosphate dehydrogenase activities, while the intestine presented higher glutathione peroxidase and glutathione reductase activities and oxidised glutathione content, which seems to reveal a higher glutathione dependency of the intestinal antioxidant response. Total and reduced glutathione contents in liver and intestine and superoxide dismutase activity in the intestine were enhanced by dietary glutamine, though lipid peroxidation values were not affected. Overall, differences between liver and intestine glutamine metabolism and antioxidant response were identified and the potential of dietary glutamine supplementation to gilthead sea bream's antioxidant response was elucidated.

1. Introduction

Glutamine (Gln) is the most prevalent amino acid (AA) in body fluids and muscle, and its turnover rates exceed that of other AA (Wu et al., 2011). Gln is also the main systemic nitrogen carrier, has a major role in ammonia detoxification and is considered a conditionally-essential AA under specific physiological conditions, such as malnutrition, infection and inflammation (Lobley et al., 2001; Wu et al., 2011). Increasing intracellular Gln concentration stimulates protein synthesis and inhibits proteolysis in muscle and enterocytes (Wu and Thompson, 1990; Xi et al., 2011). Besides its role in protein synthesis, Gln is the most versatile AA in cellular metabolism, intervening in several Gln-dependent regulatory pathways indispensable for cell proliferation, differentiation, migration and metabolism, besides being involved in purine and pyrimidine synthesis and gene expression regulation. Gln is also a major energy source for rapidly proliferating cells, including enterocytes, lymphocytes and intestinal mucosal cells (Wu et al., 2011), up-regulate antioxidant genes expression, prevents oxidant-induced apoptosis (Brasse-Lagnel et al., 2009), and modulates intestinal oxidative status, being precursor of glutathione, an important antioxidant molecule (Cheng et al., 2011).

In farm animals, stress modulation and improved immune response through dietary supplementation of Gln, has proved successful (Lobley et al., 2001; Adams and Thompson, 2006). Traditional classification of glutamine (Gln) as non-essential AA thus underestimate its functional properties in health maintenance, immune function and gastrointestinal integrity. Since Gln is extensively catabolised by enterocytes, it plays an essential role in maintaining intestinal integrity and function (Curi et al., 2005). Intestinal Gln metabolism relies extensively on

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dietary Gln intake, thus maintenance of normal intestinal physiology is critically dependent on nutritional Gln provision.

Nutrition-based health approach is a new challenge in modern aquaculture, and within this context, intestinal Gln metabolism and the role of Gln on intestinal health are of particular interest. In hybrid sturgeon (*Acipenser schrenckii* × *Huso dauricus*). Gln supplementation was reported to enhance weight gain, feed efficiency, and intestine structure and function (Qiyou et al., 2011). In Jian carp (*Cuprinus carpio* var. Jian) and channel catfish (*Ictalurus punctatus*) Gln also promoted enterocytes and leucocytes proliferation (Chen et al., 2009; Pohlenz et al., 2012a) and enhanced enterocytes glutathione biosynthesis and enzymatic antioxidant capacity in Jian carp (Chen et al., 2009; Hu et al., 2014). However, knowledge on Gln metabolism in fish, especially at the intestinal level, is still scarce and deserves further studies. Further, the potential of dietary Gln supplementation to modulate AA and glucose intestinal uptake has yet to be studied in fish. Therefore, this study was conducted to evaluate the effects of dietary Gln supplementation on performance, hepatic and intestinal Gln metabolism, and on the oxidative status and intestinal nutrient absorption capacity in gilthead sea bream, a major aquaculture species in the Mediterranean.

2. Material and methods

2.1. Diets

Four experimental diets were formulated to be isolipidic (18% lipids) and isoproteic (43% protein), with half of protein from fish meal and the other half provided by plant feedstuffs (soybean meal and wheat). Essential amino acid profile of these diets were checked to be similar to that previously estimated for gilthead sea bream (Peres and Oliva-Teles, 2009). Diets were supplemented with 0, 0.5%, 1% and 2% Gln replacing a dispensable amino acid (DAA) mixture that resembled that of gilthead sea bream whole-body AA composition (Kaushik, 1998). Ingredients and proximate composition of the experimental diets are presented in Table 1. AA composition of experimental diets is presented in Table 2.

2.2. Growth trial

This experiment followed FELASA category C recommendations and was conducted according to the European Union Directive 2010/63/EU on the protection of animals for scientific purposes. The trial was carried out at the experimental facilities of the Marine Zoology Station, Porto University, in a thermo-regulated recirculation water system equipped with 24 cylindrical fibreglass tanks of 100 L capacity. Gilthead sea bream (*Sparus aurata*) juveniles were obtained from a commercial hatchery and after transportation to the experimental facilities were submitted to a quarantine period of two weeks and then acclimated for one month to the rearing system. During this period fish were fed a commercial diet. Then, 12 groups of 25 fish with a mean body weight of 13.0 g were established and the experimental diets were randomly assigned to triplicate groups of these fish. During the trial fish were fed to apparent visual satiety twice a day, 6 days a week. Utmost care was taken to assure that all feed supplied was consumed. The trial lasted 6 weeks and during this period water temperature was maintained at 26 ± 1 °C, salinity averaged 33 ± 2‰ and dissolved oxygen was kept near saturation (7 mg L^{-1}). A natural photoperiod (12 h light and 12 h dark) was adopted.

2.3. Sampling

A random sample of 15 fish from the initial batch was taken, euthanized by lethal dose of anaesthesia (ethylene glycol monophenyl ether) and pooled for whole-body composition analysis. At the end of the trial, following one day of feed deprivation, fish from each tank were slightly anaesthetized (ethylene glycol monophenyl ether, 0.3 mL L^{-1}), bulk weighted and returned to the same tanks where they continued to be fed for two more days. Then, 6 h after the morning meal, 6 fish per tank were sacrificed by severing the spinal cord. Whole intestine was isolated and divided into pyloric caeca, proximal and distal segments according to visual dissimilarities in diameter and mucosal thickness.

Table 1
Ingredient composition and proximate analysis of the experimental diets.

	Control	0.5Gln	1Gln	2Gln
Ingredients (% dry weight)				
Fish meal[1]	24	24	24	24
CPSP[2]	5	5	5	5
Soybean meal[3]	26	26	26	26
Wheat meal[4]	13.6	13.6	13.6	13.6
Fish oil	13.6	13.6	13.6	13.6
Vitamins premix[5]	1	1	1	1
Choline chloride (50%)	0.5	0.5	0.5	0.5
Minerals premix[6]	1	1	1	1
Binder[7]	1	1	1	1
Cellulose	5.9	6.1	6.2	6.6
NEAA premix[8]	6.2	5.5	4.8	3.4
Dibasic calcium phosphate	1.2	1.2	1.2	1.2
Agar	1.0	1.0	1.0	1.0
L-glutamine	0	0.5	1	2
Proximate analysis (% dry weight)				
Dry matter (%)	86.0	90.8	86.1	86.5
Protein	44.8	44.2	43.6	44.0
Crude fat	18.2	18.8	18.5	18.5
Ash	10.7	10.8	10.8	10.5
Gross energy (kJ g^{-1})	23.0	22.6	22.5	22.2

[1] Pesquera Centinela, Steam Dried LT, Chile (CP: 72.1%; CL 9.51%). Sorgal, S.A. Ovar, Portugal.
[2] Soluble fish protein concentrate, Sopropêche, France (CP: 80.4% DM; GL: 19.7% DM).
[3] Soybean meal (CP: 51.3%; CL:2.6%), Sorgal, S.A. Ovar, Portugal.
[4] Wheat meal (CP: 14.1%; CL: 3.2%), Sorgal, S.A. Ovar, Portugal.
[5] Vitamins (mg kg^{-1} diet): retinol, 18,000 (IU kg^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.
[6] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet).
[7] Aquacube. Agil, UK.
[8] NEAA Premix composition (g 100^{-1} g DM NEAA premix): L-alanine: 19.57; L-aspartic acid: 27.95 g; L-glycine: 22.86 g; L-serine: 13.91 g; L-proline: 15.71 g.

Table 2
Determined amino acid composition (g 16 g^{-1} N) of the experimental diets[1].

	Control	0.5Gln	1Gln	2Gln
IAA[2]				
Arginine	6.75	6.57	6.30	7.08
Histidine	2.36	2.42	2.23	2.33
Isoleucine	3.47	3.64	3.37	3.61
Leucine	6.42	6.29	6.37	6.22
Lysine	6.64	6.87	6.71	6.83
Threonine	4.01	4.02	4.03	4.04
Valine	4.62	4.37	4.42	4.24
Methionine	2.31	2.31	2.33	2.30
Phenylalanine	4.00	4.18	3.90	3.95
DAA[3]				
Tyrosine	2.83	2.66	3.05	2.80
Alanine	7.39	6.66	7.60	6.14
Aspartic acid	11.89	11.49	10.67	10.04
Glutamic acid	13.06	14.16	15.42	17.78
Glycine	8.26	7.94	7.35	6.80
Serine	6.18	5.97	6.08	5.65
Proline	6.36	6.81	6.54	6.50
∑ IAA	40.6	40.7	39.7	40.6
∑ DAA	56.0	55.7	56.7	55.7
IAA/DAA	0.73	0.73	0.70	0.73

[1] Dietary tryptophan and cysteine levels were not determined.
[2] IAA, indispensable amino acids.
[3] DAA, dispensable amino acids.

Segments were opened lengthwise, washed in isosmotic saline solution containing protease inhibitor phenyl–metyl–sulphonil–fluoride (PMSF 0.2 mM), immediately frozen in liquid nitrogen and stored at −80 °C until used for the isolation of intestinal brush border membrane vesicles (BBMV; Sala-Rabanal et al., 2004). Following one day of food deprivation, other 6 fish per tank were sampled and killed by severing the spinal cord, and total, liver and viscera weights were recorded for hepatosomatic (HSI) and visceral (VI) indexes calculation. Liver and intestine (clear from adherent cells) samples were rapidly frozen in liquid nitrogen and then stored at −80 °C until further analysis. Other 3 fish per tank were killed by lethal anaesthesia for liver and whole-body composition analysis.

2.4. Analytical methods

2.4.1. Proximate composition

Chemical analysis of the experimental diets and whole-fish was conducted as follows: water content, by drying samples in an oven at 105 °C until constant weight; ash, by incineration in a muffle furnace at 450 °C for 16 h; crude protein (N × 6.25), by the Kjeldahl method after acid digestion using a Kjeltec digestion and distillation units (Tecator Systems, Höganäs, Sweden; models 1015 and 1026, respectively); crude lipids, by petroleum ether extraction (SoxTec HT System); gross energy, by direct combustion of samples in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261).

Dietary samples for AA analysis were hydrolyzed for 23 h with 6N hydrochloric acid at 110 °C under N_2 atmosphere. Samples were then derivatized with phenylisothiocyanate (PITC) reagent before separation by gradient exchange chromatography (Waters auto sample model 717 plus; Waters binary pump model 1525; Waters dual absorbance detector model 2487), according to the Pico-Tag method, as described by Cohen et al. (1989). Chromatographic peaks were identified, integrated and quantified with a Waters Breeze software package by comparing to a known AA standard (Pierce NC10180). Norleucine was used as an internal standard. Tryptophan and cysteine were not measured.

For hepatic glycogen content a portion of liver was homogenized in five volumes of iced-cold distilled water and stored at −80 °C until analysis. Glycogen content was determined by amyloglucosidase hydrolysis following the method described by Roehrig and Allred (1974). Hepatic lipids were determined gravimetrically according to Folch et al. (1957). Hepatic soluble protein concentration was determined according to Bradford (1976), using a commercial kit (Sigma protein Kit, cod. B6916) and bovine serum albumin as standard.

2.5. Enzyme activities

Liver and intestine samples (n = 9) were diluted at 1:9 and 1:4, respectively, and homogenized at pH 7.8 in ice-cold 100 mM Tris–HCl buffer containing 0.1 mM EDTA and 0.1% (v/v) Triton X-100. All procedures were performed on ice. Homogenates were centrifuged at 30,000 ×*g* for 30 min at 4 °C and the resultant supernatants were separated in aliquots and stored at −80 °C for further enzyme assays. All enzyme activities were measured at 37 °C in a Multiskan GO microplate reader (Model5111 9200; Thermo Scientific, Nanjing, China).

Glutamate dehydrogenase (GDH; EC 1.4.1.2) and glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activities were assayed as described by Morales et al. (1990). Alanine aminotransferase (ALAT, EC 2.6.1.2) and aspartate aminotransferase (ASAT, EC 2.6.1.1) activities were measured using commercial kits from Spinreact (ALAT/GPT, ref. 41283; ASAT/GOT, ref. 41273). GlNase activity was assayed similarly to Chamberlin et al. (1991). Enzyme activities were determined by monitoring the changes in absorbance of NADH or NADP at 340 nm. GSase activity was assayed similarly to Shankar and Anderson (1985). The reaction product, γ-glutamyl monohydroxamate, was measured at 540 nm.

The specific assay conditions of oxidative stress enzymes were as follows: Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured at 550 nm by the ferricytochrome C method using xanthine/xanthine oxidase as source of superoxide radicals (McCord and Fridovich, 1969). Catalase (CAT; EC 1.11.1.6) activity was determined according to Aebi (1984) by measuring the decrease of hydrogen peroxide concentration at 240 nm. Glutathione reductase (GR; EC 1.6.4.2) activity was determined at 340 nm by measuring the oxidation of NADPH as described by Morales et al. (2004). Glutathione peroxidase (GPX; EC 1.11.1.9) activity was assayed as described by Flohé and Günzler (1984). The GSSG generated by GPX was reduced by GR and NADPH consumption rate was monitored at 340 nm.

Except for SOD, for which one unit of activity was defined as the amount of enzyme necessary to produce a 50% inhibition of the ferricytochrome C reduction rate, all other enzyme activities were expressed as units (GlNase, GSase and CAT) or milliunits (GDH, ASAT, ALAT, G6PDH, GPX and GR) per milligramme of hepatic soluble protein (specific activity). One unit of enzyme activity was defined as the amount of enzyme required to transform 1 μmol of substrate per minute under the assay conditions. Protein concentration was determined according to Bradford (1976) using Sigma protein assay kit and bovine serum albumin as standard.

2.6. Lipid peroxidation

The concentration of thiobarbituric acid reacting substances was determined as marker of lipid peroxidation (LPO) following the methodology described by Buege and Aust (1978).

2.7. Total and oxidised glutathione

A portion of liver and intestine was homogenized (1:9 and 1:4, respectively) in ice-cold solution containing 1.3% 5-sulfosalicylic acid (w/v) and 10 mM HCl, and the whole procedures were done on ice to avoid glutathione oxidation. Homogenates were centrifuged at 14,000 *g* for 10 min at 4 °C and the resulting supernatants stored at −80 °C. Total glutathione (tGSH) and oxidised glutathione (GSSG) were measured following the methods described by Griffith (1980) and Vandeputte et al. (1994) with some modifications (Perez-Jimenez et al., 2012). Standard curves of reduced glutathione (GSH) and GSSG were used for tGSH and GSSG calculations, respectively. GSH level was calculated by subtracting GSSG from tGSH values. Results are expressed as nmol g^{-1} tissue. Oxidative stress index (OSI) was calculated as follows:

$$OSI = 100 \times (2\ x\ GSSG/tGSH).$$

2.8. Nutrient uptake analysis

Brush border membrane vesicles (BBMV) were prepared from pyloric caeca and distal segment (pools of 3 fish) and from proximal segments (1 fish) as described by Sala-Rabanal et al. (2004). Briefly, samples were homogenized in a hyposmotic buffer (in mM: 100 mannitol, 2 HEPES, pH 7.4, 100 Osm) to separate mucosa from the muscular layers. Selective precipitation of the basolateral and mitochondrial membranes was achieved by addition of $MgCl_2$ to a final concentration of 10 mM. Subsequent selective centrifugations enabled purification and concentration of apical enterocyte membranes, which were vesiculated (in mM: 300 mannitol, 20 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 4.08 LiN_3, pH 7.4, 320 Osm) using an insulin syringe. Alkaline phosphatase (EC 3.1.3.1) activity, which has been described as a good marker of BBMV, was determined following Weiser (1973). BBMV from pyloric caeca and proximal segments were enriched in alkaline phosphatase 7.0 (±1.8) and 9.0 (±2.8) fold, respectively, while BBMV from distal

segments showed enrichment factors below 5 and were discarded (Sala-Rabanal et al., 2004).

The capacity to absorb L-arginine, L-alanine, L-glutamine and D-glucose was determined in vesicular suspensions according to the procedure described by Santigosa et al. (2011). Vesicular suspensions were mixed with incubation buffer (in mM: 250 NaSCN, 100 mannitol, 40 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 8.16 LiN_3, 0.15 unlabelled nutrient, 0.01 ^{3}H-nutrient, 320 Osm, pH 7.4) for 5 s. The reaction was stopped by adding cold stop buffer (in mM: 300 mannitol, 20 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 4.08 LiN_3, 320 Osm, pH 7.4). The resulting mix was filtered under negative pressure through 0.22-μm cellulose nitrate filters (Millipore, Bedford MA), previously soaked in cold stop buffer. Filters were washed with stop buffer and dissolved in Filtron-X scintillation liquid (ITISA S.A., Spain). Samples were counted in a scintillation counter (Packard TRI-CARB 2100 TR) at 20 °C. Radiolabeled nutrients were purchased from Amersham Pharmacia Biotech (Barcelona, Spain).

Vesicular volume of BBMV preparations were measured according to Sala-Rabanal et al. (2004) to normalize influx values. Briefly, L-alanine retained inside the vesicles at equilibrium was measured by incubating BBMV preparation with incubation buffer. After 90 min, the reaction was stopped and the amount of L-alanine retained (vesicular retention at equilibrium) was measured as described above. Protein concentration of different vesicular suspensions was measured using BIORAD® reagent according to Bradford (1976).

2.9. Statistical analysis

Growth performance, feed utilization, body and liver composition data were analysed by one-way ANOVA. All other data was analysed by two-way ANOVA with organs or intestinal portions and diets as fixed factors. Data were checked for normal distribution and homogeneity of variances and normalized when appropriate. Significant differences among means ($P < 0.05$) were determined by the Tukey multiple range test. Statistical analysis was performed using SPSS for Windows version 22 software package.

3. Results

All experimental diets were well accepted by the fish and during the trial mortality was very low and unaffected by dietary treatments (Table 3). At the end of the growth trial, weight gain, daily growth index and feed intake were not affected by dietary treatments. Feed efficiency and protein efficiency ratio were lower in fish fed the 0.5Gln diet than the control and 1Gln diets, but no differences in nitrogen retention were observed among dietary groups (Table 3).

At the end of the trial whole-body lipids were higher and ash was lower than at the start of the trial. At the end of the trial there were no differences among groups in liver and whole-body composition nor in hepatosomatic and visceral indexes (Table 4).

Amino acid metabolism enzyme activities were different in liver and intestine (Table 5). GlNase activity was higher in the intestine than in the liver, while the opposite was true for ALAT, ASAT, GDH and GSase activities. With the exception of GSase, diet composition did not affect the activity of the amino acid metabolism enzymes measured (Table 5). GSase activity was higher in fish fed diet 0.5Gln than diets 1Gln and 2Gln.

With the exception of SOD, antioxidant enzyme activities were different in liver and intestine (Table 6). GPX and GR activities were higher in the intestine than in the liver and the opposite was true for G6PDH and CAT activities. Dietary Gln supplementation led to a decrease of GPX in both tissues.

Higher tGSH, GSH contents were observed in the liver than in the intestine, while the opposite was true for GSSG (Table 7). tGSH and GSH contents were higher in fish fed diets 0.5Gln and 1Gln than the other diets. In the intestine, but not in the liver, GSH was also higher in fish fed the 0.5Gln diet than the control. LPO and OSI were higher in the intestine than in the liver (Table 7). But, while LPO values were unaffected by diet composition, OSI was higher in the intestine of fish fed diets 0.5Gln and 2Gln than the other diets.

The volume of brush border membrane vesicles (BBMV) were similar between dietary treatments or intestinal regions (Fig. 1A). Nevertheless, to eliminate potential effect of vesicular volume variability on nutrient uptake within each treatment, influx values were expressed as intravascular concentration per second of incubation (pmols $\mu L^{-1} \cdot s^{-1}$). L-glutamine, L-arginine and D-glucose influxes were higher in proximal intestine than in pyloric caeca preparations but were not affected by dietary treatments (Fig. 1B, C, D).

4. Discussion

Previous studies showed that between 35 and 100% dietary fishmeal can be replaced by a mixture of plant-protein sources in gilthead sea bream diets without compromising growth performance (Gomez-Requeni et al., 2003; Gomez-Requeni et al., 2004;

Table 3
Growth performance and feed utilization of gilthead sea bream fed the experimental diets.

Diets	Control	0.5Gln	1Gln	2Gln	One-way ANOVA
Initial body weight (IBW), g	12.9 ± 0.002	12.9 ± 0.004	12.9 ± 0.006	12.9 ± 0.005	NS
Final body weight (FBW), g	37.3 ± 1.5	35.2 ± 0.6	37.7 ± 1.8	36.5 ± 0.8	NS
Weight gain (g kg ABW$^{-1\S}$ day^{-1})	25.5 ± 0.8	24.3 ± 0.3	25.7 ± 1	25.1 ± 0.5	NS
Feed Intake (g kg ABW$^{-1\S}$ day^{-1})	36.9 ± 2.14	40.2 ± 1.78	36.1 ± 1.38	40.1 ± 2.22	NS
Feed efficiency (g kg ABW$^{-1\S}$ day^{-1})[1]	0.69 ± 0.02 [bc]	0.61 ± 0.02 [a]	0.71 ± 0.04 [c]	0.63 ± 0.02 [ab]	*
Daily growth index (%)[2]	2.6 ± 0.12	2.5 ± 0.05	2.6 ± 0.14	2.6 ± 0.07	NS
Protein efficiency ratio[3]	1.5 ± 0.05 [bc]	1.4 ± 0.04 [a]	1.6 ± 0.10 [c]	1.4 ± 0.06 [ab]	*
Nitrogen intake (g kg ABW$^{-1\S}$ day^{-1})[4]	2.76 ± 0.16	2.85 ± 0.13	2.62 ± 0.10	2.89 ± 0.16	NS
Nitrogen retention (g kg ABW$^{-1\S}$ day^{-1})[5]	0.69 ± 0.03	0.61 ± 0.02	0.58 ± 0.09	0.64 ± 0.01	NS
Nitrogen retention (%NI)[6]	25.0 ± 1.32	21.5 ± 0.90	22.1 ± 2.98	22.1 ± 1.40	NS
Mortality (%)	4.0 ± 4.0	2.7 ± 2.3	1.3 ± 2.3	5.3 ± 4.6	NS

Values presented as mean ± S.D. (n = 3). NS: non-significant ($P \geq 0.05$).
Means with different superscript letters are significantly different ($P < 0.05$).
* $P < 0.05$.
§ ABW: average body weight (initial body weight + final body weight) / 2.
1 FE: wet weight gain/dry feed intake.
2 DGI: $(FBW^{1/3} - IBW^{1/3}) / (\text{time in days}) \times 100$.
3 PER: wet weight gain/crude protein intake.
4 NI: (N intake × 1000) / (ABW × time in days).
5 NR (g N kg^{-1} day^{-1}): (FBW × FBN) − (IBW × IBN) / (ABW × time in days).
6 NR (%NI): NR = ((FBW × FBN − IBW × IBN) / NI) × 100.

Table 4
Carcass composition (% wet weight), liver composition, hepatosomatic index (HSI) and visceral index (VI) of gilthead sea bream fed the experimental diets.

Diets	Initial	Control	0.5Gln	1Gln	2Gln	One-way ANOVA
Whole-body composition						
Dry matter (%)	28.5	29.7 ± 0.4	29.4 ± 0.4	27.5 ± 3.1	29.3 ± 0.4	NS
Ash (%)	6.94	4.4 ± 0.1	4.3 ± 0.1	3.8 ± 0.5	4.2 ± 0.1	NS
Lipid (%)	4.96	8.4 ± 0.1	7.8 ± 0.7	7.5 ± 1.1	7.7 ± 0.3	NS
Protein (%)	18.2	17.3 ± 0.3	16.7 ± 0.3	15.5 ± 1.8	16.7 ± 0.3	NS
Liver composition						
Lipids (mg g^{-1})	ND	65.2 ± 14.3	61.1 ± 4.1	71.1 ± 9.1	64.0 ± 9.9	NS
Glycogen (mg g^{-1})	ND	53.7 ± 17.7	71.0 ± 14.3	64.9 ± 17.1	63.6 ± 13.1	NS
Protein (mg g^{-1})	ND	17.4 ± 0.7	17.3 ± 0.7	17.0 ± 1.4	18.4 ± 1.4	NS
Indexes						
HSI	ND	1.4 ± 0.11	1.4 ± 0.08	1.3 ± 0.16	1.3 ± 0.07	NS
VI	ND	7.5 ± 0.15	7.7 ± 0.03	7.6 ± 0.16	7.7 ± 0.17	NS

Values presented as mean ± S.D. (n = 3). NS: non-significant (P > 0.05).

Kissil and Lupatsch, 2004; Sitja-Bobadilla et al., 2005; Watson et al., 2013; Couto et al., 2014). In fact, dietary plant-protein was even reported to improve gilthead sea bream feed utilization (Gomez-Requeni et al., 2004; Kissil and Lupatsch, 2004; Sitja-Bobadilla et al., 2005). On this basis, in the present trial a practical diet with 50% fishmeal replaced by a mixture of plant ingredients was used as control.

Growth of gilthead sea bream was high and overall performance and whole-body composition were consistent with previous studies in gilthead sea bream of the same class of age and size (Gomez-Requeni et al., 2003; Enes et al., 2008; Peres and Oliva-Teles, 2009; Perez-Jimenez et al., 2013; Couto et al., 2014). Given that Gln is not an essential amino acid, though it is considered conditionally-essential under specific physiological conditions (Lobley et al., 2001; Wu et al., 2011), it is not surprising that growth performance and whole-body composition were not affected by dietary Gln supplementation. Similar results were also previously observed in channel catfish fed Gln supplemented diets (Pohlenz et al., 2012a). In fact, protein efficiency ratio and feed efficiency were depressed, due to a slight increase of feed intake, in fish fed the 0.5Gln and 2Gln diets, but not in fish fed the 1Gln diet. Gomez-Requeni et al. (2003) also observed impaired feed conversion ratio and nitrogen retention and increased ammonia excretion in gilthead seabream fed glutamate supplemented diets as result of a lower dietary IAA/DAA ratio. However this hypothesis does not hold to explain the depress feed utilization observed in 0.5Gln and 2Gln treatments since the IAA/DAA ratio remained unaltered.

Despite the above discussion, other studies have reported positive effects of dietary Gln supplementation to feed utilization and growth performance in several fish species. For instance, in red drum (*Sciaenops ocellatus*), although growth was not improved, feed efficiency was enhanced in Gln supplemented diets (Cheng et al., 2011). In hybrid striped bass (*Morone chrysops* × *Morone saxatilis*; Cheng et al., 2012), hybrid sturgeon (Qiyou et al., 2011) and Jian carp (Yan and Qiu-Zhou, 2006) both feed utilization and weight gain improvements were reported with dietary Gln supplementation. These apparently conflicting results are not exclusive to fish (Lobley et al., 2001) and seem to reveal species specific physiological differences that still need to be fully understood (Pohlenz et al., 2012a). Moreover, the distinct diet formulation strategies used to test AA supplementation in fish may also contribute for the mention ambiguous results.

In fish, it is not yet clear if Gln is the major energy source of enterocytes and other rapidly proliferating cells, as it is the case in mammals (Yan and Qiu-Zhou, 2006; Wu et al., 2011). However, and similarly to what was observed in rat (James et al., 1998), gilthead sea bream presented a two-fold lower GSase / GlNase ratio in the intestine than in the liver, which suggests a high dependency of the intestine on an external Gln supply. This is also supported by the higher GlNase activity in the

Table 5
Specific activities of hepatic and intestinal ALAT, ASAT, GDH, GlNase and GSase of gilthead sea bream fed the experimental diets.

Organ	Liver				Intestine			
Diets	Control	0.5Gln	1Gln	2Gln	Control	0.5Gln	1Gln	2Gln
ALAT	331 ± 25	341.8 ± 17	315 ± 56	297 ± 47	78 ± 23	91 ± 39	79 ± 32	90 ± 13
ASAT	970 ± 87	936 ± 73	1004 ± 143	1009 ± 127	364 ± 51	399 ± 166	543 ± 245	397 ± 84
GDH	129 ± 20	118 ± 15	132 ± 20	127 ± 17	69 ± 10	67 ± 27	78 ± 35	86 ± 9
GlNase	1.0 ± 0.3	0.9 ± 0.2	1.2 ± 0.8	1.2 ± 0.3	1.8 ± 1.0	1.7 ± 1.2	1.9 ± 1.2	1.7 ± 1.1
GSase	5.1 ± 1.3	6.2 ± 2.2	5.9 ± 2.2	4.9 ± 1.2	4.6 ± 1.6	6.3 ± 2.6	2.5 ± 0.7	4.0 ± 1.3
Two-way ANOVA								
	Variance source				Diets			
	Organ	Diet	Interaction		Control	0.5Gln	1Gln	2Gln
ALAT	***	NS	NS		–	–	–	–
ASAT	***	NS	NS		–	–	–	–
GDH	***	NS	NS		–	–	–	–
GlNase	**	NS	NS		–	–	–	–
GSase	*	*	NS		ab	b	a	a

Values presented as mean ± S.D. (n = 9). Enzyme activities expressed as mU mg $protein^{-1}$ for ALAT, ASAT and GDH and as U mg $protein^{-1}$ for GlNase and GSase. Two-way ANOVA: NS: non-significant (P ≥ 0.05).
* P < 0.05.
** P ≤ 0.01.
*** P ≤ 0.001.

Table 6
Liver and intestine antioxidant enzymes activity of gilthead sea bream fed the experimental diets.

Organ	Liver				Intestine			
Diets	Control	0.5Gln	1Gln	2Gln	Control	0.5Gln	1Gln	2Gln
G6PDH	76.4 ± 8.3 [b]	73.8 ± 11.6 [b]	58.4 ± 12.2 [a]	73.5 ± 12.4 [ab]	9.6 ± 5	13.0 ± 7	9.8 ± 6	9.7 ± 3
CAT	896.9 ± 183.1	952.5 ± 111.2	934.0 ± 190.2	851.2 ± 92.5	62.2 ± 14	57.2 ± 12	66.7 ± 17	60.0 ± 11
GR	9.9 ± 1.5	9.9 ± 0.9	9.8 ± 1.3	9.5 ± 1.2	17.3 ± 6	12.9 ± 5	13.4 ± 5	14.8 ± 2
SOD	139.9 ± 16.1	130.8 ± 15.2	126.9 ± 22.2	129.2 ± 13.5	115 ± 15 [a]	134 ± 37 [ab]	156 ± 23 [b]	135 ± 27 [ab]
GPX	73.1 ± 10.8 [b]	55.9 ± 11.7 [a]	65.3 ± 12.2 [ab]	56.3 ± 19.7 [ab]	194 ± 42 [b]	102 ± 44 [a]	124 ± 47 [a]	143 ± 54 [ab]
Two-way ANOVA								
	Variance source				Diets			
	Organ	Diet	Interaction		Control	0.5Gln	1Gln	2Gln
G6PDH	***	**	*					
CAT	***	NS	NS		–	–	–	–
GR	***	NS	NS		–	–	–	–
SOD	NS	NS	*					
GPX	***	***	*					

Values presented as mean ± S.D. (n = 9). Enzyme activities expressed as mU mg protein^{-1} for G6PDH, GR and GPX and as U mg protein^{-1} for CAT and SOD. Two-way ANOVA: NS: non-significant (P ≥ 0.05); if interaction was significant, one-way ANOVA was performed for each organ and means in the same line with different superscript letters are significantly different (P < 0.05).
* P < 0.05.
** P ≤ 0.01.
*** P ≤ 0.001.

intestine compared to the liver, while for the other amino acid catabolism enzymes (ALAT, ASAT, GDH and GSase) the opposite was observed.

On the other hand, both in the liver and the intestine, dietary Gln supplementation had no effect in GSase and GlNase activities comparatively to fish fed the control diet. This may indicate that instead of being used to fulfil fish energy demand additional glutamine was funnelled towards other physiological purposes. For instance, it was already reported that dietary Gln supplementation increased digestive enzyme activities in Jian carp (Yan and Qiu-Zhou, 2006); increased enterocytes migration in channel catfish (Pohlenz et al., 2012a); enhanced the enteric microstructure in Jian carp (Yan and Qiu-Zhou, 2006), red drum (Cheng et al., 2011), hybrid sturgeon (Zhu et al., 2011) and channel catfish (Pohlenz et al., 2012a); improved antioxidant defence system in hybrid sturgeon (Qiyou et al., 2011; Zhu et al., 2011) and in isolated enterocytes of Jian carp (Chen et al., 2009); enhanced the innate immune system of red drum (Cheng et al., 2012), hybrid sturgeon (Zhu et al., 2011) and hybrid striped bass (Cheng et al., 2012); and improved both innate and adaptive immune responses in channel catfish (Pohlenz et al., 2012b, 2012c).

GlNase and GSase are responsible for the reversible conversion of Gln to glutamate, which is substrate for ALAT, ASAT and GDH (Wicks and Randall, 2002). As GlNase and GSase activities were unaffected by dietary Gln supplementation in both liver and intestine, no significant differences were also to be expected in the activities of these amino acid catabolism enzymes. In a previous study in gilthead sea bream Gomez-Requeni et al. (2003) has also reported few modifications on the amino acid catabolism enzymes activities in response to the dietary amino acid profile.

In fish, evidences exist of a positive effect of dietary Gln and glutamate supplementation to the glutathione content of different tissues as result of the increased glutamate provision for glutathione biosynthesis (Zhu et al., 2011; Zhao et al., 2015). In the present study, increased total glutathione (tGSH) and reduced glutathione (GSH) levels were also observed in both liver and intestine of fish fed Gln supplemented

Table 7
Liver and intestine total glutathione (tGSH), oxidised glutathione (GSSG), reduced glutathione (GSH), oxidative stress index (OSI) and lipid peroxidation (LPO) levels of gilthead sea bream fed the experimental diets.

Organ	Liver				Intestine			
Diets	Control	0.5Gln	1Gln	2Gln	Control	0.5Gln	1Gln	2Gln
tGSH	2542 ± 549	3201 ± 147	2911 ± 405	2857 ± 272	2012 ± 247	2652 ± 345.1	2338 ± 166.1	2121 ± 292.9
GSSG	91.6 ± 19.4	125.4 ± 27.6	118.3 ± 27.8	122.3 ± 42.5	223.0 ± 56 [a]	328.8 ± 89.8 [b]	165.8 ± 43.9 [a]	261.5 ± 99.9 [ab]
GSH	2451 ± 546	3075 ± 170	2792 ± 385	2735 ± 276	1789 ± 269	2323 ± 362	2172 ± 151	1859 ± 266
OSI	7.46 ± 1.89	7.90 ± 2.03	8.00 ± 1.32	8.63 ± 3.22	22.6 ± 6.8 [ab]	25.2 ± 7.5 [b]	14.1 ± 3.4 [a]	24.6 ± 9.2 [b]
LPO	12.8 ± 3.6	10.5 ± 1.4	11.4 ± 1.1	11.7 ± 1.4	21.0 ± 3	23.1 ± 2	25.1 ± 7	23.5 ± 5
Two-way ANOVA								
	Variance source				Diets			
	Organ	Diet	Interaction		Control	0.5Gln	1Gln	2Gln
tGSH	***	***	NS		a	c	bc	ab
GSSG	***	**	*					
GSH	***	***	NS		a	c	bc	ab
OSI	***	NS	*					
LPO	***	NS	NS		–	–	–	–

Values presented as mean ± S.D. (n = 9). LPO values expressed as nmols MDA g^{-1} tissue and GSH, tGSH and GSSG as nmol g^{-1} tissue. Two-way ANOVA: NS: non-significant (P ≥ 0.05); if interaction was significant, one-way ANOVA was performed for each organ and means in the same line with different superscript letters are significantly different (P < 0.05).
* P < 0.05.
** P ≤ 0.01.
*** P ≤ 0.001.

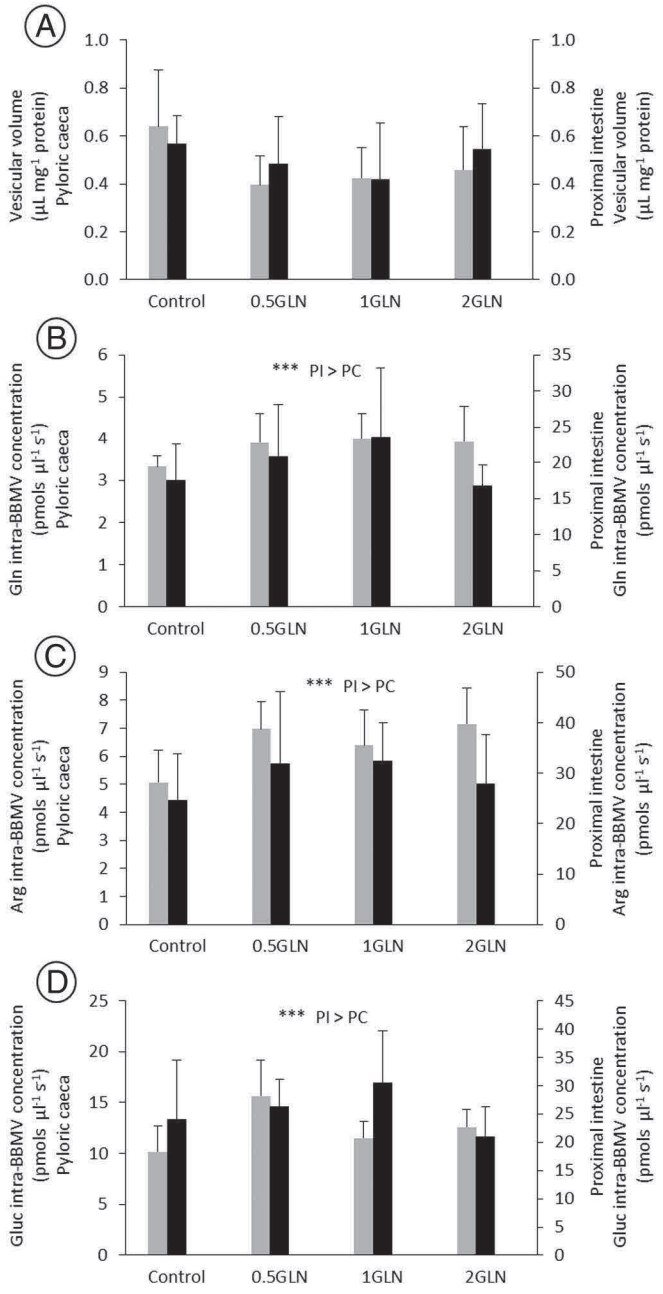

Fig. 1. Vesicular volume (A) and intravesicular L-glutamine (B), L-arginine (C) and D-glucose (D) concentration in BBMVs obtained from pyloric caeca (grey) and proximal intestine (black) of gilthead sea bream, 6 h after feeding. Values presented as mean ± S.D. (n = 6). Two-way ANOVA revealed no dietary or interaction (Diet * Intestinal portion) significant effects; *** indicate significant differences ($P < 0.001$) between pyloric caeca (PC) and proximal intestine (PI).

diets, which was particularly evident in fish fed the 0.5Gln and 1Gln diets.

Superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities were reported to increase with dietary Gln supplementation in hybrid sturgeon (Zhu et al., 2011) and Jian carp enterocytes (Jiang et al., 2009). Further, in Jian carp enterocytes cultures challenged with H_2O_2, the supplementation with Gln restored (Hu et al., 2014) or maintained (Chen et al., 2009) SOD and GPX activities. In the present study, dietary Gln supplementation also enhanced SOD activity in the intestine, but not in the liver of gilthead sea bream. On the other hand, GPX activity was depressed in both liver and intestine. The reduction of GPX activity can be explained by a decrease of H_2O_2 due to the higher GSH content, which scavenges H_2O_2 producing GSSG. This was particularly evident in the intestine of fish fed the 0.5Gln diet, which presented the lowest GPX activity and the highest GSH and GSSG contents. Despite the differences regarding the antioxidant enzymes activity and the GSH and GSSG content observed among treatments, the overall oxidative damage of both liver and intestine was similar among treatments, as shown by LPO values.

GR is responsible for restoring GSH by reducing GSSG. Even though an increase of GSSG content was observed in the intestine of fish fed the 0.5Gln diet no effect was observed in the GR activity. This may be related to the relatively low levels of GSSG comparatively to total GSH, which could be insufficient to trigger GR activity. Nonetheless, GR activity was higher in the intestine than in the liver which correlates well with the levels of GSSG in the two tissues.

Liver is a major producer and storage organ of GSH, as result of hepatocytes ability to convert methionine to cysteine and to store cysteine, a limiting amino acid for GSH biosynthesis (Lu, 2000; Ojopagogo et al., 2013). In opposition, the intestine is a major consumer of GSH, being supplied with hepatic GSH by the plasma (Wu et al., 2004). This extensive use of GSH by the intestine is related to its susceptibility to oxidative damage, due to the high turnover rates of enterocytes. This fact may explain the 2-fold higher LPO values observed in the intestine when compared to the liver.

The present study evidences some differences regarding the antioxidant defence mechanism between the liver and intestine of gilthead sea bream, as it was also previously reported for European sea bass (*Dicentrarchus labrax*; Castro et al., 2015), rainbow trout and sturgeon (Trenzado et al., 2006). Activities of GPX and GR were, respectively, 2.2- and 1.5-fold higher in the intestine than in the liver, while G6PDH and CAT activities were, respectively, 7- and 15-fold higher in the liver than in the intestine, and SOD activity was similar in both organs. As expected, tGSH and GSH contents were higher (1.3- and 1.4-fold, respectively) in the liver, while the GSSG content was higher in the intestine (2.7- and 2.1-fold, respectively). Both OSI and LPO values were also higher in the intestine than in the liver, once again denoting a higher oxidative stress in the intestine. These results also seem to reveal that the intestinal antioxidant capacity is more dependent on GSH than the liver, since GSH is extensively used both directly, as a H_2O_2 scavenger, and indirectly as substrate for GPX.

The absorption capacity study clearly evidenced higher transport capacity of the proximal intestine relatively to pyloric caeca for the three molecules tested: Gln, Arg and Glu. In rainbow trout and gilthead sea bream Santigosa et al. (2011) also observed a shift of transport capacity from the pyloric caeca to the proximal intestine when 75% of dietary fishmeal was replaced by plant protein. The authors considered this shift to represent a delay in the absorption from pyloric caeca to proximal and/or distal intestine segments, caused by an alteration of the luminal nutrient availability due to the plant protein inclusion in the diet. In the present trial diets contained only 50% of plant protein and a similar shift of transport capacity from the pyloric caeca to the proximal intestine also seems to have occurred. However, given the lack of a fishmeal-based diet in the present study it is not possible to corroborate Santigosa et al. (2011) hypothesis.

This is the first study to address the potential of dietary amino acids supplementation to regulate intestinal absorption capacity of fish. Fish glucose and amino acid transporters are considered to be similar to those found in mammals (Collie and Ferraris, 1995) and although transport might to a limited extent occur by passive diffusion and Na^+-independent transport, there is a predominance of Na^+-dependent transport (Jürss and Bastrop, 1995; Pan et al., 2004; Krogdahl et al., 2005). In rat, Gln is mainly transported by a Na^+-dependent neutral amino acid system (System B), accounting for 80–90% of total Gln absorption in the post-prandial or basal state, but also by a Na^+-independent system (System L), responsible for 10–20%, and by passive diffusion, with an almost negligible contribution (Pan et al., 2004). In fish, four Na^+-dependent amino acid carrier mechanisms were identified in eels (Cassano et al., 1990), one of which is a neutral amino acid

transport system very similar to the one found in mammalian small intestine (Hopfer, 1987). As it shares its main transporter with other neutral amino acids, Gln absortion was shown to be inhibited by L-alanine, L-serine, L-cystine and L-leucine (Fan et al., 1998; Pan et al., 2004). Gln absortion by System L may also be inhibited by L-alanine, L-serine and L-cysteine (Pan et al., 2004). In rat, high doses of dietary Gln supplementation (30% total dietary nitrogen provided by Gln) were shown to increment up to 75% the absorption capacity of the small intestine BBMV (Salloum et al., 1990). Our results, however, did not reveal any effect of dietary Gln supplementation on Gln, Arg or Glu vesicular uptake.

Similarly, Stein et al. (1987), in a study that tested dietary supplementation with seven amino acids (acidic, neutral, basic amino acids and imino acids) reported that mouse D-glucose uptake is regulated independently from dietary amino acids. The effects of each amino acids on the preferential substrates of acidic, neutral, basic amino acids and imino acids transporters were also evaluated in that study, and it was concluded that different amino acid transporters are regulated semi-independently of each other and only in a few cases a substrate was an effective inducer of its main transporter. Similar results were observed in other studies (Stein et al., 1987; Ferraris and Diamond, 1989; Salloum et al., 1990; Wu, 2013).

Thus, it is not surprising that dietary Gln supplementation levels used in the present trial did not trigger a significant increase in Gln BBMV absorption capacity, since a possible inductive effect of Gln might have been weakened by either competitive or inhibitory effects of other dietary amino acids.

In conclusion, this study showed that growth and feed utilization of gilthead sea bream juveniles was not affected by the dietary Gln supplementation levels tested. Also, dietary Gln did not modulate intestinal and hepatic GlNase and GSase enzyme activities or the intestinal absorption capacity of the animals. However, dietary Gln supplementation was shown to modulate differently hepatic and intestinal antioxidant responses, despite not altering the overall oxidative damage.

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References

Adams, A., Thompson, K.D., 2006. Biotechnology offers revolution to fish health management. Trends Biotechnol. 24, 201–205.
Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Brasse-Lagnel, C., Lavoinne, A., Husson, A., 2009. Control of mammalian gene expression by amino acids, especially glutamine. FEBS J. 276, 1826–1844.
Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Methods Enzymol. 52, 302–310.
Cassano, G., Maffia, M., Ramires, P.A., Vilella, S., Storelli, C., 1990. Do neutral amino acids and their N-methylated analogues share the same Na-dependent carrier in brush-border membrane from the eel intestine? Comp. Biochem. Physiol. A 96, 13–17.
Castro, C., Peréz-Jiménez, A., Coutinho, F., Díaz-Rosales, P., Serra, C.R., Peres, H., Oliva-Teles, A., 2015. Dietary carbohydrates and lipid source affect oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles. Br. J. Nutr. http://dx.doi.org/10.1017/S0007114515003360.
Chamberlin, M.E., Glemet, H.C., Ballantyne, J.S., 1991. Glutamine metabolism in a holostean (*Amia calva*) and teleost fish (*Salvelinus namaycush*). Am. J. Physiol. 260, 159–166.
Chen, J., Zhou, X.-Q., Feng, L., Liu, Y., Jiang, J., 2009. Effects of glutamine on hydrogen peroxide-induced oxidative damage in intestinal epithelial cells of Jian carp (*Cyprinus carpio* var. Jian). Aquaculture 288, 285–289.
Cheng, Z.Y., Buentello, A., Gatlin, D.M., 2011. Effects of dietary arginine and glutamine on growth performance, immune responses and intestinal structure of red drum, *Sciaenops ocellatus*. Aquaculture 319, 247–252.
Cheng, Z.Y., Gatlin, D.M., Buentello, A., 2012. Dietary supplementation of arginine and/or glutamine influences growth performance, immune responses and intestinal morphology of hybrid striped bass (*Morone chrysops* × *Morone saxatilis*). Aquaculture 362, 39–43.
Cohen, S.A., Meys, M., Tarvin, T., 1989. The Pico-Tag Method. A Manual of Advance Techniques for Amino Acid Analysis. Waters Chromatography Division, Milford, MA.
Collie, N.L., Ferraris, R.P., 1995. Nutrient fluxes and regulation in fish intestine. In: Mommsen, H.A. (Ed.)Biochemestry and Molecular Biology of Fishes vol. 4. Elsevier Science, Amsterdam, pp. 221–239.
Couto, A., Kortner, T.M., Penn, M., Bakke, A.M., Krogdahl, Å., Oliva-Teles, A., 2014. Effects of dietary phytosterols and soy saponins on growth, feed utilization efficiency and intestinal integrity of gilthead sea bream (*Sparus aurata*) juveniles. Aquaculture 432, 295–303.
Curi, R., Lagranha, C.J., Doi, S.Q., Sellitti, D.F., Procopio, J., Pithon-Curi, T.C., Corless, M., Newsholme, P., 2005. Molecular mechanisms of glutamine action. J. Cell. Physiol. 204, 392–401.
Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2008. Growth performance and metabolic utilization of diets with native and waxy maize starch by gilthead sea bream (*Sparus aurata*) juveniles. Aquaculture 274, 101–108.
Fan, M.Z., Adeola, O., McBurney, M.I., Cheeseman, C.I., 1998. Kinetic analysis of l-glutamine transport into porcine jejunal enterocyte brush-border membrane vesicles. Comp. Biochem. Physiol. A 121, 411–422.
Ferraris, R.P., Diamond, J.M., 1989. Specific regulation of intestinal nutrient transporters by their dietary substrates. Annu. Rev. Physiol. 51, 125–141.
Flohé, L., Günzler, W.A., 1984. Assay of glutathione peroxidase. Methods Enzymol. 105, 115–121.
Folch, J., Lees, M., Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509.
Gomez-Requeni, P., Mingarro, M., Kirchner, S., Calduch-Giner, J.A., Medale, F., Corraze, G., Panserat, S., Martin, S.A.M., Houlihan, D.F., Kaushik, S.J., Perez-Sanchez, J., 2003. Effects of dietary amino acid profile on growth performance, key metabolic enzymes and somatotropic axis responsiveness of gilthead sea bream (*Sparus aurata*). Aquaculture 220, 749–767.
Gomez-Requeni, P., Mingarro, M., Calduch-Giner, J.A., Medale, F., Martin, S.A.M., Houlihan, D.F., Kaushik, S., Perez-Sanchez, J., 2004. Protein growth performance, amino acid utilisation and somatotropic axis responsiveness to fish meal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*). Aquaculture 232, 493–510.
Griffith, O.W., 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal. Biochem. 106, 207–212.
Hopfer, V., 1987. Membrane transport mechanisms for hexoses and amino acids in the small intestine. In: Johnson, L.R. (Ed.), Physiology of the Gastrointestinal Tract. Raven Press, New York, pp. 1499–1526.
Hu, K., Feng, L., Jiang, W., Liu, Y., Jiang, J., Li, S., Zhou, X., 2014. Oxidative damage repair by glutamine in fish enterocytes. Fish Physiol. Biochem. 40, 1437–1445.
James, L.A., Lunn, P.G., Elia, M., 1998. Glutamine metabolism in the gastrointestinal tract of the rat assessed by the relative activities of glutaminase (EC 3.5.1.2) and glutamine synthetase (EC 6.3.1.2). Br. J. Nutr. 79, 365–372.
Jiang, J., Zheng, T., Zhou, X.Q., Liu, Y., Feng, L., 2009. Influence of glutamine and vitamin E on growth and antioxidant capacity of fish enterocytes. Aquac. Nutr. 15, 409–414.
Jürss, K., Bastrop, R., 1995. Amino acid metabolism in fish. In: Hochachka, P.W., Mommsen, T.P. (Eds.), Biochemistry and Molecular Biology of Fishes. Elsevier, pp. 159–189.
Kaushik, S.J., 1998. Whole body amino acid composition of European sea bass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and turbot (*Psetta maxima*) with an estimation of their IAA requirement profiles. Aquat. Living Resour. 11, 355–358.
Kissil, G.W., Lupatsch, I., 2004. Successful replacement of fishmeal by plant proteins in diets for the gilthead seabream, *Sparus aurata* L. Isr. J. Aquacult. Bamidgeh 56, 188–199.
Krogdahl, A., Hemre, G.I., Mommsen, T.P., 2005. Carbohydrates in fish nutrition: digestion and absorption in postlarval stages. Aquac. Nutr. 11, 103–122.
Lobley, G.E., Hoskin, S.O., McNeil, C.J., 2001. Glutamine in animal science and production. J. Nutr. 131, 2525S–2531S.
Lu, S.C., 2000. Regulation of glutathione synthesis. Curr. Top. Cell. Regul. 36, 95–116.
McCord, J.M., Fridovich, I., 1969. Superoxide dismutase: an enzymic function for erythrocuprein. J. Biol. Chem. 244, 6049–6055.
Morales, A.E., Garciarejon, L., Delahiguera, M., 1990. Influence of handling and/or anesthesia on tress response in Rainbow-trout — effects on liver primary metabolism. Comp. Biochem. Physiol. A 95, 87–93.
Morales, A.E., Perez-Jimenez, A., Hidalgo, M.C., Abellan, E., Cardenete, G., 2004. Oxidative stress and antioxidant defenses after prolonged starvation in *Dentex dentex* liver. Comp. Biochem. Physiol. C 139, 153–161.
Ojopagogo, Y.A., Adewale, I.O., Afolayan, A., 2013. Preliminary studies on the renaturation of denatured catfish (*Clarias gariepinus*) glutathione transferase. Fish Physiol. Biochem. 39, 1657–1663.
Pan, M., Meng, Q., Choudry, H.A., Karinch, A.M., Lin, C., Souba, W.W., 2004. Stimulation of intestinal glutamine absorption in chronic metabolic acidosis. Surgery 136, 127–134.
Peres, H., Oliva-Teles, A., 2009. The optimum dietary essential amino acid profile for gilthead seabream (*Sparus aurata*) juveniles. Aquaculture 296, 81–86.

Perez-Jimenez, A., Peres, H., Rubio, V.C., Oliva-Teles, A., 2012. The effect of hypoxia on intermediary metabolism and oxidative status in gilthead sea bream (*Sparus aurata*) fed on diets supplemented with methionine and white tea. Comp. Biochem. Physiol. C 155, 506–516.

Perez-Jimenez, A., Peres, H., Rubio, V.C., Oliva-Teles, A., 2013. Effects of diet supplementation with white tea and methionine on lipid metabolism of gilthead sea bream juveniles (*Sparus aurata*). Fish Physiol. Biochem. 39, 661–670.

Pohlenz, C., Buentello, A., Bakke, A.M., Gatlin, D.M., 2012a. Free dietary glutamine improves intestinal morphology and increases enterocyte migration rates, but has limited effects on plasma amino acid profile and growth performance of channel catfish *Ictalurus punctatus*. Aquaculture 370, 32–39.

Pohlenz, C., Buentello, A., Criscitiello, M.F., Mwangi, W., Smith, R., Gatlin, D.M., 2012b. Synergies between vaccination and dietary arginine and glutamine supplementation improve the immune response of channel catfish against *Edwardsiella ictaluri*. Fish Shellfish Immunol. 33, 543–551.

Pohlenz, C., Buentello, A., Mwangi, W., Gatlin, D.M., 2012c. Arginine and glutamine supplementation to culture media improves the performance of various channel catfish immune cells. Fish Shellfish Immunol. 32, 762–768.

Qiyou, X., Qing, Z., Hong, X., Chang'an, W., Dajiang, S., 2011. Dietary glutamine supplementation improves growth performance and intestinal digestion/absorption ability in young hybrid sturgeon (*Acipenser schrenckii* female × *Huso dauricus* male). J. Appl. Ichthyol. 27, 721–726.

Roehrig, K.L., Allred, J.B., 1974. Direct enzymatic procedure for the determination of liver glycogen. Anal. Biochem. 58, 414–421.

Sala-Rabanal, M., Gallardo, M.A., Sánchez, J., Planas, J.M., 2004. Na-dependent-glucose transport by intestinal brush border membrane vesicles from gilthead sea bream (*Sparus aurata*). J. Membr. Biol. 201, 85–96.

Salloum, R.M., Souba, W.W., Fernandez, A., Stevens, B.R., 1990. Dietary modulation of small intestinal glutamine transport in intestinal brush border membrane vesicles of rats. J. Surg. Res. 48, 635–638.

Santigosa, E., Garcia-Meilan, I., Valentin, J.M., Perez-Sanchez, J., Medale, F., Kaushik, S., Gallardo, M.A., 2011. Modifications of intestinal nutrient absorption in response to dietary fish meal replacement by plant protein sources in sea bream (*Sparus aurata*) and rainbow trout (*Onchorynchus mykiss*). Aquaculture 317, 146–154.

Shankar, R.A., Anderson, P.M., 1985. Purification and properties of glutamine synthetase from liver of *Squalus acanthias*. Arch. Biochem. Biophys. 239, 248–259.

Sitja-Bobadilla, A., Pena-Llopis, S., Gomez-Requeni, P., Medale, F., Kaushik, S., Perez-Sanchez, J., 2005. Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). Aquaculture 249, 387–400.

Stein, E.D., Chang, S.D., Diamond, J.M., 1987. Comparison of different dietary amino acids as inducers of intestinal amino acid transport. Am. J. Physiol. 252, 626–635.

Trenzado, C., Hidalgo, M.C., Garcia-Gallego, M., Morales, A.E., Furne, M., Domezain, A., Domezain, J., Sanz, A., 2006. Antioxidant enzymes and lipid peroxidation in sturgeon *Acipenser naccarii* and trout *Oncorhynchus mykiss*. Aquaculture 254, 758–767.

Vandeputte, C., Guizon, I., Genestie-Denis, I., Vannier, B., Lorenzon, G., 1994. A microtiter plate assay for total glutathione and glutathione disulfide contents in cultured/isolated cells: performance study of a new miniaturized protocol. Cell Biol. Toxicol. 10, 415–421.

Watson, A.M., Barrows, T.F., Place, A.R., 2013. Taurine supplemented plant protein based diets with alternative lipid sources for juvenile gilthead sea bream, *Sparus aurata*. J. Int. Fish. Aquaculture 4.

Weiser, M.M., 1973. Intestinal epithelial cell surface membrane glycoprotein synthesis. An indicator of cellular differentiation. J. Biol. Chem. 248, 2536–2546.

Wicks, B.J., Randall, D.J., 2002. The effect of sub-lethal ammonia exposure on fed and unfed rainbow trout: the role of glutamine in regulation of ammonia. Comp. Biochem. Physiol. A 132, 275–285.

Wu, G., 2013. Protein Digestion and Absorption of Peptides and Amino Acids. Amino Acids. CRC Press, Boca Raton 33–62.

Wu, G.Y., Thompson, J.R., 1990. The effect of glutamine on protein turnover in chick skeletal muscle in vitro. Biochem. J. 265, 593–598.

Wu, G.Y., Fang, Y.Z., Yang, S., Lupton, J.R., Turner, N.D., 2004. Glutathione metabolism and its implications for health. J. Nutr. 134, 489–492.

Wu, G., Bazer, F.W., Johnson, G.A., Knabe, D.A., Burghardt, R.C., Spencer, T.E., Li, X.L., Wang, J.J., 2011. Triennial growth symposium: important roles for L-glutamine in swine nutrition and production. J. Anim. Sci. 89, 2017–2030.

Xi, P., Jiang, Z., Zheng, C., Lin, Y., Wu, G., 2011. Regulation of protein metabolism by glutamine: implications for nutrition and health. Front. Biosci. 16, 578–597.

Yan, L., Qiu-Zhou, X., 2006. Dietary glutamine supplementation improves structure and function of intestine of juvenile Jian carp (*Cyprinus carpio* var. Jian). Aquaculture 256, 389–394.

Zhao, Y., Hu, Y., Zhou, X.Q., Zeng, X.Y., Feng, L., Liu, Y., Jiang, W.D., Li, S.H., Li, D.B., Wu, X.Q., Wu, C.M., Jiang, J., 2015. Effects of dietary glutamate supplementation on growth performance, digestive enzyme activities and antioxidant capacity in intestine of grass carp (*Ctenopharyngodon idella*). Aquac. Nutr.

Zhu, Q., Xu, Q.Y., Xu, H., Wang, C.A., Sun, D.J., 2011. Dietary glutamine supplementation improves tissue antioxidant status and serum non-specific immunity of juvenile hybrid sturgeon (*Acipenser schrenckii* female × *Huso dauricus* male). J. Appl. Ichthyol. 27, 715–720.

Chapter 3

Dietary arginine surplus does not improve intestinal nutrient absorption capacity, amino acid metabolism and oxidative status of gilthead sea bream (*Sparus aurata*) juveniles

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Dietary arginine surplus does not improve intestinal nutrient absorption capacity, amino acid metabolism and oxidative status of gilthead sea bream (*Sparus aurata*) juveniles

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ABSTRACT

This study aims to evaluate the effects of dietary arginine (Arg) surplus on gilthead sea bream growth, intestinal nutrient absorption capacity, hepatic and intestinal amino acid (AA) metabolism, and oxidative status. For that purpose a 6 weeks growth trial was performed with gilthead sea bream juveniles fed four isolipidic (18%) and isoproteic (44%) diets supplemented with 0, 0.5, 1 and 2% Arg. Dietary Arg did not affect fish growth performance, feed and nitrogen utilization, body composition, and activity of the main AA catabolic enzymes in liver and intestine. Similarly, intestinal nutrient absorption capacity, evaluated by intestinal brush border membrane vesicles technique, was also unaffected by the dietary Arg surplus. On the other hand, enzymatic antioxidant response was modulated by dietary Arg increment, which led to a reduced glutathione peroxidase activity in the liver and the intestine and increased superoxide dismutase activity in the intestine, but did not affect the overall lipid peroxidation values. Some differences in liver and intestine antioxidant enzymatic responses were identified, with the liver showing higher catalase and glucose-6-phosphate dehydrogenase activities, while the intestine presented higher glutathione peroxidase and glutathione reductase activities. Overall, dietary Arg excess showed limited potential to enhance gilthead sea bream performance and intestinal nutrient absorption capacity, but it was shown to modulate hepatic and intestinal antioxidant defences, without affecting overall lipid peroxidation.

Statement of relevance: Recently some fish species have been shown to benefit from dietary arginine surplus, while evidences also exist of an Arg-Lys antagonism in other fish species. This manuscript gives further insight on the potential of dietary arginine supplementation in gilthead sea bream, a major aquaculture species in the Mediterranean.

1. Introduction

In the past decades a great deal of research focused on the development of more sustainable aquafeeds. It is now feasible to formulate diets for several carnivorous fish species with substantial amount of protein of plant origin without impairing growth performance (Oliva-Teles et al., 2015). For instance, gilthead sea bream (*Sparus aurata*), a major aquaculture species in the Mediterranean, has recently been shown to tolerate a 100% plant protein diet without compromising growth (Kissil and Lupatsch, 2004; Watson et al., 2013).

However, even if no decreased performance or pathological signs are observed in fish fed plant protein-based diets, physiological alterations may still occur potentially impairing fish immunity and stress responses. In fact, intestinal inflammation and depressed immune status due to the anti-nutrients found in plant feedstuffs were reported in gilthead sea bream fed plant feedstuff-rich diets (Bonaldo et al., 2008; Kokou et al., 2012; Sitja-Bobadilla et al., 2005). Moreover, disturbances of the intestinal mucosal structure have also been identified in gilthead sea bream fed diets including purified saponins and phytosterols, anti-nutritional factors present in soybean among other feedstuffs (Couto et al., 2014). This, associated with the stressful environment of intensive

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culture conditions and the increasing farmers and consumers concerns with fish health and welfare, has driven attention to the potential of nutritional-based health approaches (Kiron, 2012; Oliva-Teles, 2012; Pohlenz and Gatlin Iii, 2014).

Arginine (Arg) is an indispensable amino acid (IAA) that, besides being required for protein synthesis, is the most abundant nitrogen carrier for tissue proteins and is also essential for the synthesis of biologically important molecules such as proline, creatine, ornithine, polyamines, and nitric oxide (NO) (Wu and Morris, 1998). Moreover, in fish given high Arg doses either by injection or through force-feeding, Arg was shown to be a more potent insulinotropic than glucose, leading to increasing titres of insulin (Andoh, 2007; Baños et al., 1999; Lall et al., 1994; Mommsen et al., 2001; Plisetskaya et al., 1991; Sink and Lochmann, 2007), insulin-like growth factor 1 (IGF-1), and growth hormone (GH) (Baños et al., 1999; Lall et al., 1994; Mommsen et al., 2001).

This remarkable endocrine modulatory capacity of Arg has been pointed as the main cause for the enhanced growth performance and protein efficiency observed in channel catfish (*Ictalurus punctatus*) (Pohlenz et al., 2014), juvenile Atlantic salmon (*Salmo salar*) (Andersen et al., 2013) and hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) (Cheng et al., 2012) fed diets with an Arg surplus. However, results on dietary Arg surplus effects on fish performance are diverse, with studies in black seabream (*Acanthopagrus schlegelii*) (Zhou et al., 2012a), European sea bass (Tulli et al., 2007), Japanese flounder (*Paralichthys olivaceus*) (Alam et al., 2002), red drum (*Sciaenops ocellatus*) (Cheng et al., 2011), grouper (*Epinephelus coioides*) (Luo et al., 2007) and adult Atlantic salmon (Andersen et al., 2014) reporting that dietary Arg surplus did not change growth performance and feed utilization, while studies in rainbow trout (*Oncorhynchus mykiss*) (Kaushik and Fauconneau, 1984; Kaushik et al., 1988) and blunt snout bream (*Megalobrama amblycephala*) (Ren et al., 2013) reported that dietary Arg excess resulted in growth depression, which was associated to an Arg-lysine (Lys) antagonism. This antagonism is yet to be fully understood in fish, although it was suggested to result from competition between Arg and Lys at intestinal transporters (Berge et al., 1999; Kaushik et al., 1988), or to an increased ureagenesis (Kaushik and Fauconneau, 1984). In fact, since the majority of teleosts are ammoneotelic and lack a fully functional ornithine–urea cycle (Ip and Chew, 2010; Ip et al., 2001), argininolysis is of major importance for the overall urea production (Fournier et al., 2003).

Evidences also exist of a protective role of dietary Arg against cell membranes oxidative damage by reactive oxygen species (ROS) (Ren et al., 2013). Wang et al. (2015) suggested that dietary Arg decreases oxidative damage by elevating fish radical scavenging ability, although further studies are required to confirm this hypothesis and to fully understand the potential of dietary Arg supplementation to mitigate fish oxidative stress. Thus, the present work was performed to evaluate the effects of a dietary Arg surplus on gilthead sea bream performance, hepatic and intestinal AA metabolism, and oxidative status. Additionally, an evaluation of the effect of dietary Arg excess on intestinal L-Arg, L-glutamine and D-glucose absorption capacity was performed for the first time in fish.

2. Material and methods

2.1. Diets

Four isolipidic (18%) and isonitrogenous (7%) diets were formulated with an amino acid profile similar to that of the whole-body of gilthead sea bream (Kaushik, 1998). Diets were supplemented with 0, 0.5%, 1% and 2% Arg at the expense of a dispensable amino acid (DAA) mixture. Ingredients and proximate composition of the experimental diets are presented in Table 1 and the AA composition of the experimental diets is presented in Table 2.

Table 1
Ingredient composition and chemical analysis of the experimental diets.

	Control	0.5Arg	1Arg	2Arg
Ingredients (% dry weight)				
Fish meal[a]	24	24	24	24
CPSP G[b]	5	5	5	5
Soybean meal[c]	26	26	26	26
Whole wheat meal[d]	13.6	13.6	13.6	13.6
Fish oil	13.6	13.6	13.6	13.6
Vitamin premix[e]	1	1	1	1
Choline chloride (50%)	0.5	0.5	0.5	0.5
Minerals premix[f]	1	1	1	1
Binder[g]	1	1	1	1
Cellulose	5.9	6.5	7.2	8.4
NEAA premix[h]	6.2	5.0	3.9	1.6
Dibasic calcium phosphate	1.2	1.2	1.2	1.2
Agar	1	1	1	1
L-Arginine	0	0.5	1	2
Chemical analysis (% dry weight)				
Dry matter (%)	86.0	87.1	88.1	91.9
Crude protein	45.0	44.4	44.4	43.1
Crude lipid	18.2	18.3	18.0	16.6
Ash	10.7	10.6	10.5	10.3
Gross energy (kJ g^{-1})	23.0	22.6	22.5	21.8

[a] Pesquera Centinela, Steam Dried LT, Chile (CP: 72.1%; CL 9.51%). Sorgal, S.A. Ovar, Portugal.
[b] Soluble fish protein concentrate, Sopropêche, France (CP: 80.4% DM; GL: 19.7% DM).
[c] Soybean meal (CP: 51.3%; CL: 2.6%), Sorgal, S.A. Ovar, Portugal.
[d] Whole wheat meal (CP: 14.1%; CL: 3.2%), Sorgal, S.A. Ovar, Portugal.
[e] Vitamins (mg kg^{-1} diet): retinol, 18,000 (IU kg^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.
[f] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet).
[g] Aquacube, (Guar gum, polymethyl carbamide, Manioc starch blend, hydrate calcium sulphate). Agil, England.
[h] NEAA Premix composition (g 100^{-1} g DM NEAA premix): L-alanine: 19.57; L-aspartic acid: 27.95 g; L-glycine: 22.86 g; L-serine: 13.91 g; L-proline: 15.71 g.

2.2. Growth trial

This feeding trial was carried out at the Marine Zoology Station, Porto University, experimental facilities, in a thermo-regulated water semi-recirculation system equipped with 24 cylindrical fiberglass

Table 2
Determined amino acid composition (g 16 g^{-1}N) of the experimental diets[a].

	Control	0.5Arg	1Arg	2Arg
IAA[b]				
Arginine	6.8	8.0	9.3	11.4
Histidine	2.4	2.7	2.5	2.9
Isoleucine	3.5	3.7	3.8	3.8
Leucine	6.4	6.5	6.7	6.4
Lysine	6.6	6.5	6.2	6.4
Threonine	4.0	4.0	4.1	4.1
Valine	4.6	4.4	4.5	4.5
Methionine	2.3	2.4	2.4	2.3
Phenylalanine	4.0	4.0	4.0	4.1
DAA[c]				
Tyrosine	2.8	3.0	3.2	3.7
Alanine	7.4	7.1	6.8	6.4
Aspartic acid	11.9	11.6	11.2	10.2
Glutamic acid	13.1	13.6	13.7	13.5
Glycine	8.3	7.9	7.2	6.8
Serine	6.2	6.4	6.0	5.7
Proline	6.4	6.8	6.3	6.1
$\sum$ IAA	40.6	42.2	43.4	45.9
$\sum$ DAA	56.0	56.4	54.3	52.4
IAA/DAA	0.73	0.75	0.80	0.88

[a] Dietary tryptophan and cysteine levels were not determined.
[b] IAA, indispensable amino acids.
[c] DAA, dispensable amino acids.

tanks of 100 L capacity. The growth trail followed FELASA category C recommendations and was conducted according to the European Union Directive 2010/63/EU on the protection of animals for scientific purposes. Gilthead sea bream juveniles obtained from a commercial hatchery (Mariscos de Estero S.A., Huelva, Spain) were submitted to a quarantine period of two weeks after the arrival to the experimental facilities and were then acclimated for one month to the rearing system and fed a commercial diet (Aquagold 3 mm, Aquasoja, Soja de Portugal, S.A). Then, 12 groups of 25 fish each with a mean body weight of 13.0 g were established and the experimental diets randomly assigned to triplicate groups of these fish. The trial lasted 6 weeks, during which the fish were fed twice a day to apparent visual satiety, 6 days a week. Utmost care was taken to assure that all feed supplied was consumed. The water temperature was maintained at 26 ± 1 °C, salinity averaged 33 ± 2‰ and a photoperiod of 12 h light and 12 h dark was adopted.

2.3. Sampling

Fifteen fish from the initial batch were randomly sampled, euthanized by a lethal dose of anaesthesia (ethylene glycol monophenyl ether) and pooled for whole-body composition analysis. At the end of the trial, following one day of feed deprivation, fish from each tank were slightly anaesthetized (ethylene glycol monophenyl ether, 0.3 ml l^{-1}), bulk weighted, and returned to the same tanks. Fish were fed for two more days and then, 6 h after the morning meal, 6 fish per tank were sacrificed by severing the spinal cord. Whole intestine was isolated and divided into pyloric caeca, proximal and distal intestine. Distal intestine was distinguished according to visual dissimilarities in diameter and mucosal thickness. Segments were opened lengthwise, washed in isosmotic saline solution containing protease inhibitor phenyl-methyl-sulphonyl-fluoride (PMSF 0.2 mM), immediately frozen in liquid nitrogen and stored at −80 °C until used for the isolation of intestinal brush border membrane vesicles (BBMV) according to Sala-Rabanal et al. (2004). Following a further day of feed deprivation, 6 fish per tank were sampled and euthanized, by severing the spinal cord, and whole-fish, liver and viscera weights were recorded for hepatosomatic (HSI) and visceral (VI) indices calculation. After sampling, liver and intestine (clear of adherent cells) were rapidly frozen in liquid nitrogen and then stored at −80 °C until determination of enzyme activities. Three other fish per tank were euthanized by lethal anaesthesia for liver and whole-body composition analysis.

2.4. Analytical methods

2.4.1. Chemical composition

Analysis of the experimental diets and whole-fish were done as follows: dry matter, by drying samples in an oven at 105 °C until constant weight; ash, by incineration in a muffle furnace at 450 °C for 16 h; crude protein content (N × 6.25) by the Kjeldahl method, after acid digestion using Kjeltec digestion and distillation units (Tecator Systems, Höganäs, Sweden; models 1015 and 1026, respectively); crude lipid, by petroleum ether extraction (SoxTec HT System), and gross energy by direct combustion of samples in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261).

Dietary samples for AA analysis were hydrolyzed for 23 h with 6 N hydrochloric acid at 112 °C under N_2 atmosphere. Samples were then derivatized with phenylisothiocyanate (PITC) reagent before separation by gradient exchange chromatography (Waters auto sample model 717 plus; Waters binary pump model 1525; Waters dual absorbance detector model 2487), according to the Pico-Tag method as described by Cohen et al. (1989). Chromatographic peaks were identified, integrated and quantified with a Waters Breeze software package by comparing to known protein hydrolysate AA standards (Pierce NC10180). Norleucine was used as internal standard. Tryptophan and cysteine were not measured.

For hepatic glycogen content, a portion of liver was homogenized in five volumes of ice-cold distilled water and stored at −80 °C until analysis. Glycogen content was determined by amyloglucosidase hydrolysis following the method described by Roehrig and Allred (1974). Hepatic lipids were determined gravimetrically according to Folch et al. (1957). Hepatic soluble protein concentration was determined according to Bradford (1976), using a commercial kit (Sigma protein Kit, cod. B6916) and bovine serum albumin as standard.

2.4.2. Enzyme activities

Samples of liver and intestine (n = 9) were diluted at 1:9 and 1:4, respectively, and homogenized at pH 7.8 in ice-cold 100 mM Tris–HCl buffer containing 0.1 mM EDTA and 0.1% (v/v) Triton X-100. All procedures were performed on ice. Homogenates were centrifuged at 30,000 × *g* for 30 min at 4 °C and the resultant supernatants were separated in aliquots and stored at −80 °C for further enzyme assays. All enzyme activities were measured at 37 °C in a Multiskan GO microplate reader (Model5111 9200; Thermo Scientific, Nanjing,China).

Glutamate dehydrogenase (GDH; EC 1.4.1.2) and glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activities were assayed as described by Morales et al. (1990). Alanine aminotransferase (ALAT, EC 2.6.1.2) and aspartate aminotransferase (ASAT, EC 2.6.1.1) activities were measured using commercial kits from Spinreact, Spain (ALAT/GPT, ref. 41283; ASAT/GOT, ref. 41273). Enzyme activities were determined by monitoring the changes in absorbance of NADH or NADP at 340 nm.

Oxidative stress enzymes were analysed as follows: Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured at 550 nm by the ferricytochrome C method using xanthine/xanthine oxidase as source of superoxide radicals (McCord and Fridovich, 1969). Catalase (CAT; EC 1.11.1.6) activity was determined according to Aebi (1984) by measuring the decrease of hydrogen peroxide concentration at 240 nm. Glutathione reductase (GR; EC 1.6.4.2) activity was determined at 340 nm by measuring the oxidation of NADPH as described by Morales et al. (2004). Glutathione peroxidase (GPX; EC 1.11.1.9) activity was assayed as described by Flohé and Günzler (1984). The GSSG generated by GPX was reduced by GR and NADPH consumption rate was monitored at 340 nm.

For SOD, one unit of enzyme activity was defined as the amount of enzyme necessary to produce 50% inhibition of the ferricytochrome C reduction rate. All other enzyme activities were expressed as units (CAT) or milliunits (GDH, ASAT, ALAT, G6PDH, GPX and GR) per milligram of hepatic soluble protein (specific activity). One unit of enzyme activity was defined as the amount of enzyme required to transform 1 μmol of substrate per minute under the assay conditions. Protein concentration was determined according to Bradford (1976) as described above.

2.4.3. Lipid peroxidation

Liver and intestine concentrations of thiobarbituric acid reacting substances was determined as marker of lipid peroxidation (LPO) following the methodology described by Buege and Aust (1978).

2.4.4. Nutrient uptake

Brush border membrane vesicles (BBMV) were prepared from pyloric caeca and distal segment (pools of 3 fish) and from proximal segments (1 fish) as described by Sala-Rabanal et al. (2004). Briefly, samples were homogenized in hyposmotic buffer (in mM: 100 mannitol, 2 HEPES, pH 7.4, 100 Osm) to separate mucosa from the muscular layers. Selective precipitation of basolateral and mitochondrial membranes was achieved by addition of $MgCl_2$ to a final concentration of 10 mM. Subsequent selective centrifugations enabled purification and concentration of apical enterocyte membranes, which were vesiculated (in mM: 300 mannitol, 20 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 4.08 LiN_3, pH 7.4, 320 Osm) using an insulin syringe (BD MicroFine 1.0 ml Insulin Syringe 29G). Alkaline phosphatase (EC 3.1.3.1) activity, which has been

described as a good marker of BBMV, was determined following Weiser (1973). BBMV from pyloric caeca and proximal segments were enriched in alkaline phosphatase 9.3 (±2.6) and 7.6 (±2.4) fold, respectively, while BBMV from distal segments showed enrichment factors below 5 and were discarded (Sala-Rabanal et al., 2004).

The capacity to absorb L-arginine, L-alanine and L-glutamine and D-glucose were determined in vesicular suspensions according to the procedure described by Santigosa et al. (2011). Vesicular suspensions were mixed with incubation buffer (in mM: 250 NaSCN, 100 mannitol, 40 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 8.16 LiN_3, 0.15 unlabeled nutrient, 0.01 ^{3}H-nutrient, 320 Osm, pH 7.4) for 5 s. The reaction was stopped by adding cold stop buffer (in mM: 300 mannitol, 20 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 4.08 LiN_3, 320 Osm, pH 7.4). The resulting mix was rapidly passed under negative pressure through 0.22 μm cellulose nitrate filters (Millipore, Bedford MA), previously soaked in cold stop buffer. Filters were washed with stop buffer and dissolved in Filtron-X scintillation liquid (ITISA S.A., Spain). Samples were counted in a scintillation counter (Packard TRI-CARB 2100 TR) at 20 °C. Radiolabeled nutrients were purchased from Amersham Pharmacia Biotech (Barcelona, Spain).

Vesicular volume of BBMV preparations were measured according to Sala-Rabanal et al. (2004) to normalize influx values. Briefly, L-alanine retained inside the vesicles at equilibrium situation was measured by incubating BBMV preparation with incubation buffer. After 90 min, the reaction was stopped and the amount of L-alanine retained (vesicular retention at equilibrium) was measured as described above. Protein concentration of different vesicular suspensions was measured using BIORAD ® reagent according to Bradford (1976).

2.5. Statistical analysis

Growth performance, feed utilization, body and liver composition data were analysed by one-way ANOVA. All other data was analysed by two-way ANOVA with organs or intestinal segments and diets as fixed factors. Data were checked for normal distribution and homogeneity of variances and normalized when appropriate. Significant differences among means ($P < 0.05$) were determined by the Tukey's multiple range test. Statistical analysis was performed using a SPSS for Windows version 22 software package.

3. Results

All diets were well accepted by the fish and during the trial mortality was low and not affected by dietary treatments (Table 3). Weight gain, daily growth index, feed intake, and nitrogen retention were not affected by dietary treatments (Table 3). At the end of the trial whole-body lipids were higher and ash content was lower than at the beginning of the trial. Liver and whole-body composition, hepatosomatic and visceral indexes were similar among groups (Table 4). The activities of amino acid catabolism enzymes were higher in the liver than in the intestine (Table 5) but were not affected by diet (Table 5).

With the exception of SOD, antioxidant enzymes activities and LPO values were different in liver and intestine (Table 6). GPX and GR activities and LPO values were higher in the intestine than in the liver and the opposite was true for G6PDH and CAT activities. Dietary Arg supplementation led to a decrease of GPX activity in both tissues and to an enhancement of SOD activity in the intestine (Table 6).

BBMV vesicular volumes were not affected by dietary treatments and intestinal segments (Fig. 1). To eliminate potential effect of vesicular volume variability on nutrient uptake within each treatment, AA influx values were expressed as intra-vesicular concentration per second of incubation (pmols μL^{-1} s^{-1}). L-Glutamine, L-Arginine and D-Glucose concentrations in BBMVs were higher in proximal intestine than in pyloric caeca preparations but were not affected by dietary treatments (Fig. 1).

4. Discussion

The high growth performance attained in the present study corroborates that gilthead sea bream can cope with high fishmeal replacement by plant-protein sources without adverse effects on growth once the IAA requirements are fulfilled (Couto et al., 2014; Gomez-Requeni et al., 2003, 2004; Kissil and Lupatsch, 2004; Sitja-Bobadilla et al., 2005; Watson et al., 2013). Dietary supply of an IAA at the requirement level for a given species guarantees that maximum growth and nitrogen retention will not be compromised by that specific IAA (Bureau and Encarnação, 2006; Harper, 1956; Kaushik and Seiliez, 2010; Tibaldi and Kaushik, 2005). Thus, dietary supplementation of an IAA above the requirement level should not further enhance growth performance or nitrogen retention (Kaushik and Seiliez, 2010). In this sense, it is not surprising that in the present study gilthead sea bream growth, feed utilization, and nitrogen balance were not affected by dietary Arg levels above estimated requirements (Kaushik, 1998; Peres and Oliva-Teles, 2009). Similar results were observed in other species, such as in black sea bream juveniles (Zhou et al., 2012a), European sea bass fingerlings (Tibaldi et al., 1994) and juveniles (Tulli et al., 2007), Japanese flounder (Alam et al., 2002), red drum juveniles (Cheng et al., 2011), grouper juveniles (Luo et al., 2007) and adult Atlantic salmon (Andersen et al., 2014). On the contrary, dietary Arg surplus improved growth and feed efficiency in hybrid striped bass (Cheng et al., 2012), red drum (Cheng et al., 2011), channel catfish (Pohlenz et al., 2013, 2014) and juvenile Atlantic salmon (Andersen et al., 2013). This enhanced performance was suggested to result from the strong endocrine modulatory capacity of

Table 3
Growth performance and feed utilization of gilthead sea bream fed the experimental diets.

Diets	Control	0.5Arg	1Arg	2Arg	One-way ANOVA
Final body weight (g)	37.3 ± 1.5	37.9 ± 1.6	36.6 ± 0.7	35.2 ± 2.1	NS
Weight gain (g kgABW^{-1} day^{-1})	25.5 ± 0.8	25.8 ± 0.8	25.1 ± 0.4	24.3 ± 1.3	NS
Feed intake (g kgABW^{-1} day^{-1})	36.9 ± 2.14	36.2 ± 1.25	37.5 ± 4.29	36.2 ± 1.19	NS
Feed efficiency[a]	0.69 ± 0.02	0.69 ± 0.02	0.71 ± 0.05	0.67 ± 0.07	NS
Daily growth index[b]	2.61 ± 0.12	2.66 ± 0.12	2.55 ± 0.05	2.44 ± 0.17	NS
Protein efficiency ratio[c]	1.5 ± 0.05	1.6 ± 0.11	1.5 ± 0.15	1.6 ± 0.05	NS
Nitrogen intake (g kgABW^{-1} day^{-1})[d]	2.8 ± 0.16	2.6 ± 0.09	2.7 ± 0.31	2.5 ± 0.08	NS
Nitrogen retention (g kgABW^{-1} day^{-1})[e]	0.69 ± 0.03	0.65 ± 0.03	0.61 ± 0.04	0.63 ± 0.07	NS
Nitrogen retention (%NI)[f]	25.0 ± 1.32	24.6 ± 1.98	22.8 ± 4.12	25.1 ± 2.23	NS
Mortality (%)	4.0 ± 4.0	1.3 ± 2.3	4.0 ± 4.0	2.7 ± 2.3	NS

Values presented as mean ± S.D. (n = 3). NS: non-significant ($P \geq 0.05$).
ABW: average body weight (initial body weight, IBW + final body weight, FBW) / 2.
[a] FE: wet weight gain / dry feed intake.
[b] DGI: (FBW1/3 − IBW1/3) / time in days) × 100.
[c] PER: wet weight gain / crude protein intake.
[d] NI: (N intake × 1000) / (ABW × time in days).
[e] NR (g kg^{-1} day^{-1}): (FBW × FBN) − (IBW × IBN) / (ABW × time in days); FBN and IBN: final and initial whole-body N content, respectively.
[f] NR (%NI): NR = ((FBW × FBN − IBW × IBN) / N intake) × 100.

Table 4
Whole-body composition (% wet weight), liver composition (mg g^{-1} wet weight), hepatosomatic index (HSI) and visceral index (VI) of gilthead sea bream fed the experimental diets.

Diets	Initial	Control	0.5Arg	1Arg	2Arg	One-way ANOVA
Whole-body composition						
Dry matter	28.5	29.7 ± 0.4	29.5 ± 0.57	29.0 ± 1.18	29.2 ± 0.56	NS
Ash	6.94	4.4 ± 0.1	4.4 ± 0.26	4.1 ± 0.33	4.2 ± 0.29	NS
Lipid	4.96	8.4 ± 0.1	8.4 ± 0.95	8.7 ± 0.45	8.2 ± 0.72	NS
Protein	18.2	17.3 ± 0.3	16.6 ± 0.13	16.2 ± 0.82	16.9 ± 0.63	NS
Liver composition						
Lipids	ND	65.2 ± 14.3	65.2 ± 14.3	69.8 ± 6.2	77.7 ± 15.2	NS
Glycogen	ND	53.7 ± 17.7	57.2 ± 21.6	58.5 ± 17.0	48.3 ± 18.7	NS
Protein	ND	17.4 ± 0.7	18.5 ± 1.7	18.2 ± 1.2	18.9 ± 1.8	NS
Indexes						
HSI	ND	1.4 ± 0.11	1.4 ± 0.02	1.3 ± 0.19	1.3 ± 0.14	NS
VI	ND	7.5 ± 0.15	8.0 ± 0.07	7.7 ± 0.69	7.7 ± 0.34	NS

Values presented as mean ± S.D. (n = 3). ND: not determined. NS: non-significant (P ≥ 0.05).

Arg (Andersen et al., 2013; Pohlenz et al., 2014; Tulli et al., 2007). However, the potential beneficial effects of dietary Arg surplus in fish are yet to be elucidated. In fact, it was demonstrated in fish that Arg induces the release of glucagon (Vega-Rubín de Celis et al., 2004), somatostatin (Eilertson and Sheridan, 1995), GH and IGF-1 (Baños et al., 1999; Lall et al., 1994), and insulin (Andoh, 2007; Baños et al., 1999; Lall et al., 1994; Mommsen et al., 2001; Plisetskaya et al., 1991; Sink and Lochmann, 2007), besides being a precursor of ornithine, a rate-limiting AA for polyamine biosynthesis (Andersen et al., 2013). However, studies in Atlantic salmon also reported that a moderate dietary Arg surplus did not affect plasma insulin or GH levels (Lall et al., 1994; Pohlenz et al., 2013).

Conceivable species-specific differences in the ability to synthetize Arg (Fournier et al., 2002; Lin et al., 2015) and in the extent by which Arg is used in multiple synthetic processes (Wu et al., 2011) may help to explain the different effects of dietary Arg surplus on fish growth performance observed in different studies. For instance, although the enzyme pyrroline-5-carboxylate (P5C) reductase was previously shown to be present in the liver and intestine of rainbow trout (Dabrowski et al., 2005) little is yet known about the proline–ornithine–arginine pathway in fish, by which proline is synthetized from Arg via the arginase, ornithine aminotransferase, and P5C reductase. The absence of specific dose-response Arg requirement data has also been pointed as a possible cause for the different results observed in studies evaluating dietary Arg surplus effects (Fournier et al., 2002; Pohlenz et al., 2014; Zhou et al., 2010). In fact, Arg requirement studies in fish are still scarce (NRC, 2011) and available data may be affected by a number of factors, such as the methodological approach (AA deletion, factorial AA requirement, ideal protein concept), the dietary protein sources used, the AA levels tested, fish life stage, the response criteria (growth, feed efficiency, nitrogen or AA retention), and the mathematical model used (Bureau and Encarnação, 2006; Cowey, 1995; Shearer, 2000; Zhou et al., 2010). Accordingly, differences in Arg requirements have been reported, being Arg the IAA with the highest variation in dietary requirement among species (Fournier et al., 2002; NRC, 2011). For instance, Berge et al. (2002) concluded that Arg and Lys requirement levels for Atlantic salmon were 20% lower than earlier estimates (Berge et al., 1997, 1998). On the other hand, Buentello and Gatlin (2000) reported that Arg requirements of channel catfish were 33% higher when glycine was used to replace glutamic acid in the diets.

Evidences of adverse effects to growth performance and feed efficiency related to dietary Arg surplus also exist for a number of fish species, such as golden pompano (*Trachinotus ovatus*) (Lin et al., 2015), rainbow trout (Fournier et al., 2003; Kaushik and Fauconneau, 1984; Kaushik et al., 1988), cobia (*Rachycentron canadum*) (Van Nguyen et al., 2014), silver perch (*Bidyanus bidyanus*) (Ngamsnae et al., 1999), hybrid Clarias (*Clarias gariepinus* × *Clarias microcephalus*) (Singh and Khan, 2007), Nile tilapia (*Oriochromis niloticus*) (Santiago and Lovell, 1988), black sea bream (Zhou et al., 2011), yellow grouper (*Epinephelus awoara*) (Zhou et al., 2012a), blunt snout bream (Ren et al., 2013) and Indian major carp (*Labeo rohita*) (Abidi and Khan, 2009). In some of these studies, the negative effects of dietary Arg excess were suggested to result from an antagonism between dietary Arg and Lys levels (Berge et al., 1999, 2002; Kaushik et al., 1988; Santiago and Lovell, 1988; Zhou et al., 2011). Although an Arg-Lys antagonism is yet to be fully demonstrated in fish, evidences of reduced intestinal Lys uptake due to excess Arg exist for rainbow trout (Kaushik et al., 1988), Atlantic salmon (Berge et al., 1999) and blunt snout bream (Ren et al., 2013). It was further suggested that this Arg-Lys antagonism occurs mainly at the post-absorptive metabolic level (Davies et al., 1997; Kaushik and Fauconneau, 1984), similarly to what is known to occur in rats and poultry (Luo et al., 2004). In fact dietary Lys surplus was reported to reduce urea production in fish, as result of a Lys inhibitory action on arginase, blocking the Arg hydrolysis to ornithine and urea (Kaushik and Fauconneau, 1984, Berge et al., 2002). Dietary Arg supplementation circumvents this inhibition (Berge et al., 1998, 2002). This also agrees with the linear

Table 5
Specific activities (mU mg protein − 1) of hepatic and intestinal ALAT, ASAT and GDH of gilthead sea bream fed the experimental diets.

Organ	Liver				Intestine			
Diets	Control	0.5Arg	1Arg	2Arg	Control	0.5Arg	1Arg	2Arg
ALAT	331 ± 25	298 ± 84	273 ± 87	299 ± 59	78 ± 23	91 ± 26	65 ± 40	82 ± 12
ASAT	970 ± 87	915 ± 110	958 ± 164	965 ± 98	364 ± 51	454 ± 79	437 ± 149	433 ± 152
GDH	129 ± 20	121 ± 12	125 ± 43	118 ± 8	69 ± 10	77 ± 24	60 ± 43	79 ± 20

Two-way ANOVA

	Variance source			Diets			
	Organ	Diet	Interaction	Control	0.5Arg	1Arg	2Arg
ALAT	***	NS	NS	–	–	–	–
ASAT	***	NS	NS	–	–	–	–
GDH	***	NS	NS	–	–	–	–

Values presented as mean ± S.D. (n = 9). Two-way ANOVA: NS: non-significant (P ≥ 0.05); ***P < 0.001.

Table 6
Liver and intestine antioxidant enzymes activity and lipid peroxidation (LPO) levels of gilthead sea bream fed the experimental diets.

Organ	Liver				Intestine			
Diets	Control	0.5Arg	1Arg	2Arg	Control	0.5Arg	1Arg	2Arg
G6PDH	76.4 ± 8.3	78.7 ± 14	75.8 ± 11.6	64.1 ± 6.4	9.6 ± 5.1	8.8 ± 5.8	9.8 ± 11.0	12.3 ± 7.6
CAT	897 ± 183	836 ± 94	880 ± 182	832 ± 152	62.2 ± 14	59.5 ± 14	62.7 ± 12	66.1 ± 11
GR	9.9 ± 1.5	9.4 ± 0.6	9.8 ± 1.3	8.9 ± 1.0	17.3 ± 5.7	12.1 ± 1.8	20.2 ± 10.3	15.7 ± 4.8
SOD	140 ± 16	121 ± 25	126 ± 30	125 ± 21	115 ± 15 a	116 ± 33 a	187 ± 60 b	149 ± 38 ab
GPX	73.1 ± 10.8	59.1 ± 16.3	63.2 ± 10.7	67.9 ± 6.7	194 ± 42	139 ± 39	120 ± 65	153 ± 36
LPO	12.8 ± 3.6	11.5 ± 0.8	12.7 ± 2.6	11.3 ± 1.9	21.0 ± 3.1	20.6 ± 2.6	23.4 ± 6.6	17.8 ± 2.6

Two-way ANOVA

	Variance source			Diets			
	Organ	Diet	Interaction	Control	0.5Arg	1Arg	2Arg
G6PDH	***	NS	*				
CAT	***	NS	NS	–	–	–	–
GR	***	NS	NS	–	–	–	–
SOD	NS	*	**				
GPX	***	**	NS	b	a	a	ab
LPO	***	NS	NS	–	–	–	–

Values presented as mean ± S.D. (n = 9). Enzyme activities expressed as mU mg protein^{-1} for G6PDH, GR and GPX, as U mg protein^{-1} for CAT and SOD and as nmols MDA g^{-1} tissue for LPO. Two-way ANOVA: NS: non-significant ($P \geq 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; if interaction was significant, one-way ANOVA was performed for each organ and means in the same line with different superscript letters are significantly different ($P < 0.05$).

correlation observed between dietary Arg intake and urea-N excretion as result of an increased argininolysis (Fournier et al., 2003; Gouillou-Coustans et al., 2002).

In studies where no antagonism between Arg and Lys was evidenced, some negative effects of dietary Arg surplus were still observed and were reported to result from a toxic effect of the dietary Arg excess, and to be related to extra energy expenditure toward deamination and ammonia excretion (Ren et al., 2013; Zhou et al., 2012b; Zhou et al., 2015). In the present study, however, gilthead sea bream showed identical growth performance and liver and whole-body composition despite the different Arg-Lys ratio tested (between 1.0 and 1.8), which suggest that no Arg-Lys antagonism or Arg toxic effect should have occurred. These results are similar to what was reported for juvenile grouper (Luo et al., 2007), Nile tilapia (Yue et al., 2015), Japanese flounder (Alam et al., 2002) fed dietary Arg above the requirement level.

Dietary Arg surplus did not affect GDH, ALAT and ASAT activities, both in liver and intestine, which is in accordance with the similar whole-body nitrogen retention of the different groups. Accordingly, diets with an Arg surplus led to increased hepatic arginase activity and urea excretion in European sea bass but did not altered hepatic GDH activity (Tulli et al., 2007). The role of dietary protein level and IAA profile on the regulation of the main AA catabolism enzymes has been studied in some fish species. Some authors reported a limited responsiveness in gilthead sea bream (Coutinho et al., 2016; Gomez-Requeni et al., 2003) and in other fish species (Guerreiro et al., 2012; Peres and Oliva-Teles, 2008), while other authors reported an adaptive response to either dietary protein level (Coutinho et al., 2015; Fournier et al., 2003) or IAA profile (Fournier et al., 2003; Peres and Oliva-Teles, 2005, 2006, 2007). Further, the higher activities of the key enzymes involved in AA catabolism observed in the present study in the liver comparatively to the intestine of gilthead sea bream confirm the liver as major organ of AA metabolism (Cowey and Walton, 1989).

Recently, dietary Arg below optimum level for grass carp was shown to increase muscle protein oxidation and lipid peroxidation, as consequence of a decrease of GSH content and CAT, GPX and SOD activities and gene expression (Wang et al., 2015). Although the pathways by which dietary Arg modulates the antioxidant enzymes activity is still not fully understood, Wang et al. (2015) observed that dietary Arg supplementation was positively correlated with the expression of several antioxidant-related signalling molecules, being NO and Nrf2 the most relevant. Similarly, the maintenance of muscle GSH content was also shown to be partially dependent on Arg regulation of the glutamate-cysteine ligase (GCL) expression, a rate-limiting enzyme for GSH biosynthesis (Wang et al., 2015). Despite these evidences, it is still unclear how a dietary Arg excess affects fish oxidative status. While a dietary Arg surplus decreased blood malondialdehyde (MDA) concentration and SOD, CAT, and GPX activities in yellow catfish (Zhou et al., 2015), in grass carp it increased muscle MDA and protein carbonyl content and decreased CAT and SOD activity and GSH content (Wang et al., 2015). In the present study, dietary Arg also modulated gilthead sea bream enzymatic antioxidant response, by reducing GPX activity in the liver and intestine and by increasing SOD activity in the intestine. However, the overall LPO in both tissues was not affected by dietary Arg level.

Oxidative damage in the intestine was higher than in the liver, as evidenced by the higher LPO values observed in the intestine. This confirms previous results in this species (Coutinho et al., 2016) as well as in European sea bass (Castro et al., 2015), rainbow trout and sturgeon (Trenzado et al., 2006). GPX and GR activities, which are dependent on reduced and oxidised GSH provision, respectively, were higher in the intestine than in the liver, while the liver showed higher G6PDH and CAT activities. These results confirm that gilthead sea bream intestinal antioxidant capacity seems more dependent on GSH than the liver as previously observed by Coutinho et al. (2016).

The evaluation of intestinal absorption capacity of D-Glu, L-Arg, and L-Gln confirms a regionalization of intestinal nutrient transporters, with absorption capacity being higher in the proximal intestine than in the pyloric caeca. This agrees with previous observations in gilthead sea bream (Coutinho et al., 2016; Santigosa et al., 2011), but it is contrary to what was reported for rainbow trout, cod, largemouth bass, and striped bass (Buddington and Diamond, 1987). According to Santigosa et al. (2011) the higher absorption capacity in the proximal intestine may indicate a delay of nutrient absorption from the pyloric caeca to the proximal intestine as result of high plant protein inclusion in the diets. However, the absence of a fishmeal-based control diet in the present study does not allow to corroborate this hypothesis.

In fish, as in mammals, glucose and AA are predominantly transported by Na^+-dependent transport through the intestinal brush border membane, although nutrients may also be transported, to a limitted extent, by passive diffusion or Na^+-independent transport (Collie and Ferraris, 1995; Jürss and Bastrop, 1995; Krogdahl et al., 2005; Pan et al., 2004). Evidences exist of four different Na^+-dependent

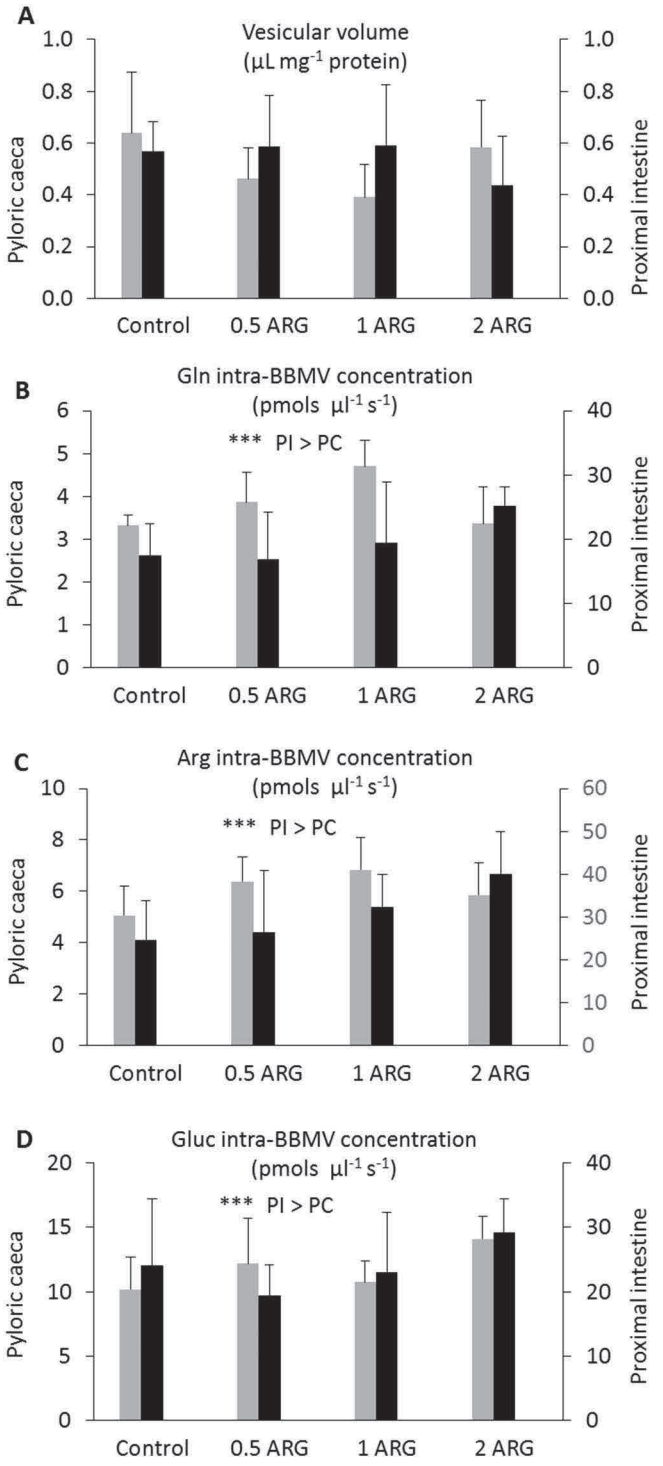

Fig. 1. Vesicular volume (A) and intravesicular L-Glutamine (B), L-arginine (C) and D-glucose (D) concentration in BBMVs obtained from pyloric caeca (grey) and proximal intestine (black) of gilthead sea bream, 6 h after feeding. Values presented as mean ± S.D. (n = 6).Two-way ANOVA revealed no dietary or interaction (Diet*Intestinal segment) significant effects; *** indicate significant differences ($P < 0.001$) between pyloric caeca (PC) and proximal intestine (PI).

AA transporters in fish, which are specific for acidic, neutral, and basic amino acids, and for imino acids (Cassano et al., 1990). Despite the apparent specificity of the Na^+-dependent AA transporters, studies in mammals revealed that the majority of AA are not effective inducers of their specific main transporters. For instance Arg, which is a basic AA, is the second-best inducer of acidic AA transporters, while aspartate, an acidic AA, is the best inducer of basic AA transporters (Ferraris and Diamond, 1989; Salloum et al., 1990; Stein et al., 1987; Wu, 2013). Moreover, several AA share the same transporter but present distinct affinities to that transporter, consequently some AA may inhibit other AA transporter. This is the case of Gln, which transport is inhibited by L-alanine, L-serine, L-cysteine and L-leucine (Fan et al., 1998; Pan et al., 2004). In European sea bass cross-inhibition experiments revealed that glycine, alanine, and methionine share the same Na^+-dependent transporter, and mutual inhibition of alanine and methionine transport by glycine occurs (Balocco et al., 1993).

Given the complexity of AA transporters regulation by dietary AA, it is not surprising that in the present study no alteration of the BBMVs L-Arg and L-Gln absorption capacity was observed independently of the dietary Arg level tested. Similarly, neither an improvement of Arg uptake nor an antagonism between Arg-Lys was observed in a recent study investigating the intestinal Arg-Lys interaction in Pacific Bluefin tuna (*Thunnus orientalis*), regardless of the Arg levels tested (Martinez-Montano et al., 2013). The fact that dietary Arg did not modify BBMVs D-glucose absorption capacity was also to be expected since evidences in mouse indicate that D-glucose transport through the intestinal brush border membrane is not regulated by dietary AA (Stein et al., 1987). These results are in line with a previous study in gilthead sea bream that showed that dietary Gln supplementation also did not modify L-Gln, L-Arg and D-glucose uptake by BBMVs (Coutinho et al., 2016).

In summary, results of the present study suggest that dietary Arg excess has limited potential to enhance growth performance, feed utilization, and D-glucose, L-Arg and L-Gln intestinal absorption capacity of gilthead sea bream juveniles. Moreover, dietary Arg modulated hepatic and intestinal enzymatic antioxidant responses, although lipid peroxidation was not affected.

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References

Abidi, S.F., Khan, M.A., 2009. Dietary arginine requirement of fingerling Indian major carp, *Labeo rohita* (Hamilton) based on growth, nutrient retention efficiencies, RNA/DNA ratio and body composition. J. Appl. Ichthyol. 25, 707–714.

Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.

Alam, M.S., Teshima, S.I., Ishikawa, M., Koshio, S., 2002. Effects of dietary arginine and lysine levels on growth performance and biochemical parameters of juvenile Japanese flounder *Paralichthys olivaceus*. Fish. Sci. 68, 509–516.

Andersen, S.M., Holen, E., Aksnes, A., Ronnestad, I., Zerrahn, J.E., Espe, M., 2013. Dietary arginine affects energy metabolism through polyamine turnover in juvenile Atlantic salmon (*Salmo salar*). Br J Nutr. 110, 1968–1977.

Andersen, S.M., Holen, E., Aksnes, A., Rønnestad, I., Zerrahn, J.E., Espe, M., 2014. Adult Atlantic salmon (*Salmo salar* L.) adapts to long-term surplus dietary arginine supplementation. Aquac. Nutr. 21, 355–363.

Andoh, T., 2007. Amino acids are more important insulinotropins than glucose in a teleost fish, barfin flounder (*Verasper moseri*). Gen. Comp. Endocrinol. 151, 308–317.

Balocco, C., Bogé, G., Roche, H., 1993. Neutral amino acid transport by marine fish intestine: role of the side chain. J. Comp. Physiol. B. 163, 340–347.

Baños, N., Planas, J.V., Gutierrez, J., Navarro, I., 1999. Regulation of plasma insulin-like growth factor-I levels in brown trout (*Salmo trutta*). Comp. Biochem. Physiol. C Toxicol. Pharmacol. 124, 33–40.

Berge, G.E., Lied, E., Sveier, H., 1997. Nutrition of Atlantic Salmon (*Salmo salar*) - the requirement and metabolism of arginine. Comp. Biochem. Physiol. A Comp. Physiol. 117, 501–509.

Berge, G.E., Sveier, H., Lied, E., 1998. Nutrition of Atlantic salmon (*Salmo salar*); the requirement and metabolic effect of lysine. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 120, 477–485.

Berge, G.E., Bakke-McKellep, A.M., Lied, E., 1999. In vitro uptake and interaction between arginine and lysine in the intestine of Atlantic salmon (*Salmo salar*). Aquaculture 179, 181–193.
Berge, G.E., Sveier, H., Lied, E., 2002. Effects of feeding Atlantic salmon (*Salmo salar* L.) imbalanced levels of lysine and arginine. Aquac. Nutr. 8, 239–248.
Bonaldo, A., Roem, A.J., Fagioli, P., Pecchini, A., Cipollini, I., Gatta, P.P., 2008. Influence of dietary levels of soybean meal on the performance and gut histology of gilthead sea bream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax* L.). Aquac. Res. 39, 970–978.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Buddington, R.K., Diamond, J.M., 1987. Pyloric ceca of fish: a "new" absorptive organ. Am. J. Physiol. Gastrointest. Liver Physiol. 252, G65–G76.
Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Methods Enzymol. 52, 302–310.
Buentello, J.A., Gatlin, D.M., 2000. The dietary arginine requirement of channel catfish (*Ictalurus punctatus*) is influenced by endogenous synthesis of arginine from glutamic acid. Aquaculture 188, 311–321.
Bureau, D.P., Encarnação, P., 2006. Adequately defining the amino acid requirements of fish: the case example of lysine. In: Cruz Suarez, L.E., Ricque Marie, D., Tapia Salazar, M., Nieto López, M.G., Villarreal, D., Puello Cruz, A.C., Garcia Ortega, A. (Eds.), Avances en Nutricion Acuicola VIII. Universidad Autonoma de Nuevo Léon, Monterrey, Nuevo Leon, México, pp. 29–54.
Cassano, G., Maffia, M., Ramires, P.A., Vilella, S., Storelli, C., 1990. Do neutral amino acids and their N-methylated analogues share the same Na-dependent carrier in brush-border membrane from the eel intestine? Comp. Biochem. Physiol. A Comp. Physiol. 96, 13–17.
Castro, C., Perez-Jimenez, A., Coutinho, F., Diaz-Rosales, P., Serra, C.A., Panserat, S., Corraze, G., Peres, H., Oliva-Teles, A., 2015. Dietary carbohydrate and lipid sources affect differently the oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles. Br. J. Nutr. 114, 1584–1593.
Cheng, Z.Y., Buentello, A., Gatlin, D.M., 2011. Effects of dietary arginine and glutamine on growth performance, immune responses and intestinal structure of red drum, *Sciaenops ocellatus*. Aquaculture 319, 247–252.
Cheng, Z.Y., Gatlin, D.M., Buentello, A., 2012. Dietary supplementation of arginine and/or glutamine influences growth performance, immune responses and intestinal morphology of hybrid striped bass (*Morone chrysops* × *Morone saxatilis*). Aquaculture 362, 39–43.
Cohen, S.A., Meys, M., Tarvin, T., 1989. The Pico-tag Method. A Manual of Advance Techniques for Amino Acid Analysis. Waters Chromatography Division, Milford, MA.
Collie, N.L., Ferraris, R.P., 1995. Nutrient fluxes and regulation in fish intestine. In: Mommsen, H.a. (Ed.), Biochemestry and Molecular Biology of Fishes. Elsevier, Amsterdam, pp. 221–239.
Coutinho, F., Peres, H., Castro, C., Perez-Jimenez, A., Pousao-Ferreira, P., Oliva-Teles, A., Enes, P., 2015. Metabolic responses to dietary protein/carbohydrate ratios in zebra sea bream (*Diplodus cervinus*, Lowe, 1838) juveniles. Fish Physiol. Biochem. (Epublication ahead of print version).
Coutinho, F., Castro, C., Rufino-Palomares, E., Ordonez-Grande, B., Gallardo, M.A., Oliva-Teles, A., Peres, H., 2016. Dietary glutamine supplementation effects on amino acid metabolism, intestinal nutrient absorption capacity and antioxidant response of gilthead sea bream (*Sparus aurata*) juveniles. Comp. Biochem. Physiol. A 191, 9–17.
Couto, A., Kortner, T.M., Penn, M., Bakke, A.M., Krogdahl, Å., Oliva-Teles, A., 2014. Effects of dietary phytosterols and soy saponins on growth, feed utilization efficiency and intestinal integrity of gilthead sea bream (*Sparus aurata*) juveniles. Aquaculture 432, 295–303.
Cowey, C.B., 1995. Protein and amino acid requirements - a critique of methods. J. Appl. Ichthyol. 11, 199–204.
Cowey, C.B., Walton, M.J., 1989. Intermediary metabolism. In: Halver, J.E. (Ed.), Fish Nutrition, second ed. Academic Press, New York, NY, pp. 260–329.
Dabrowski, K., Terjesen, B.F., Zhang, Y.F., Phang, J.M., Lee, K.J., 2005. A concept of dietary dipeptides: a step to resolve the problem of amino acid availability in the early life of vertebrates. J. Exp. Biol. 208, 2885–2894.
Davies, S.J., Morris, P.C., Baker, R.T.M., 1997. Partial substitution of fish meal and full-fat soya bean meal with wheat gluten and influence of lysine supplementation in diets for rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquac. Res. 28, 317–328.
Eilertson, C.D., Sheridan, M.A., 1995. Pancreatic somatostatin-14 and somatostatin-25 release in rainbow trout is stimulated by glucose and arginine. Am. J. Phys. 269, R1017–R1023.
Fan, M.Z., Adeola, O., McBurney, M.I., Cheeseman, C.I., 1998. Kinetic analysis of L-glutamine transport into porcine jejunal enterocyte brush-border membrane vesicles. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 121, 411–422.
Ferraris, R.P., Diamond, J.M., 1989. Specific regulation of intestinal nutrient transporters by their dietary substrates. Annu. Rev. Physiol. 51, 125–141.
Flohé, L., Günzler, W.A., 1984. Assay of glutathione peroxidase. Methods Enzymol. 105, 115–121.
Folch, J., Lees, M., Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509.
Fournier, V., Gouillou Coustans, M.F., Metailler, R., Vachot, C., Guedes, M.J., Tulli, F., Oliva-Teles, A., Tibaldi, E., Kaushik, S.J., 2002. Protein and arginine requirements for maintenance and nitrogen gain in four teleosts. Br. J. Nutr. 87, 459–469.
Fournier, V., Gouillou-Coustans, M.F., Metailler, R., Vachot, C., Moriceau, J., Le Delliou, H., Huelvan, C., Desbruyeres, E., Kaushik, S.J., 2003. Excess dietary arginine affects urea excretion but does not improve N utilisation in rainbow trout *Oncorhynchus mykiss* and turbot *Psetta maxima*. Aquaculture 217, 559–576.
Gomez-Requeni, P., Mingarro, M., Kirchner, S., Calduch-Giner, J.A., Medale, F., Corraze, G., Panserat, S., Martin, S.A.M., Houlihan, D.F., Kaushik, S.J., Perez-Sanchez, J., 2003. Effects of dietary amino acid profile on growth performance, key metabolic enzymes and somatotropic axis responsiveness of gilthead sea bream (*Sparus aurata*). Aquaculture 220, 749–767.
Gomez-Requeni, P., Mingarro, M., Calduch-Giner, J.A., Medale, F., Martin, S.A.M., Houlihan, D.F., Kaushik, S., Perez-Sanchez, J., 2004. Protein growth performance, amino acid utilisation and somatotropic axis responsiveness to fish meal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*). Aquaculture 232, 493–510.
Gouillou-Coustans, M.F., Fournier, V., Metailler, R., Vachot, C., Desbruyeres, E., Huelvan, C., Moriceau, J., Le Delliou, H., Kaushik, S.J., 2002. Dietary arginine degradation is a major pathway in ureagenesis in juvenile turbot (*Psetta maxima*). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 132, 305–319.
Guerreiro, I., Peres, H., Castro-Cunha, M., Oliva-Teles, A., 2012. Effect of temperature and dietary protein/lipid ratio on growth performance and nutrient utilization of juvenile Senegalese sole (*Solea senegalensis*). Aquac. Nutr. 18, 98–106.
Harper, A.E., 1956. Amino acid imbalance, toxicities and antagonisms. Nutr. Rev. 14, 225–227.
Ip, Y.K., Chew, S.F., 2010. Ammonia production, excretion, toxicity, and defense in fish: A review. Front. Physiol. 1, 1–20.
Ip, Y.K., Chew, S.F., Randall, D.J., 2001. Ammonia toxicity, tolerance, and excretion. In: Wright, P.A., Anderson, P.M. (Eds.), Fish Physiology. Academic Press, San Diego, pp. 109–148.
Jürss, K., Bastrop, R., 1995. Amino acid metabolism in fish. In: Hochachka, P.W., Mommsen, T.P. (Eds.), Biochemistry and Molecular Biology of Fishes. Elsevier, Amsterdam, pp. 159–189.
Kaushik, S.J., 1998. Whole body amino acid composition of European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and turbot (*Psetta maxima*) with an estimation of their IAA requirement profiles. Aquat. Living Resour. 11, 355–358.
Kaushik, S.J., Fauconneau, B., 1984. Effects of lysine administration on plasma arginine and on some nitrogenous catabolites in rainbow trout. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 79, 459–462.
Kaushik, S.J., Seiliez, I., 2010. Protein and amino acid nutrition and metabolism in fish: Current knowledge and future needs. Aquac. Res. 41, 322–332.
Kaushik, S.J., Fauconneau, B., Terrier, L., Gras, J., 1988. Arginine requirement and status assessed by different biochemical indices in rainbow trout (*Salmo gairdneri* R.). Aquaculture 70, 75–95.
Kiron, V., 2012. Fish immune system and its nutritional modulation for preventive health care. Anim. Feed Sci. Technol. 173, 111–133.
Kissil, G.W., Lupatsch, I., 2004. Successful replacement of fishmeal by plant proteins in diets for the gilthead seabream, *Sparus aurata* L. Isr. J. Aquacult. Bamidgeh 56, 188–199.
Kokou, F., Rigos, G., Henry, M., Kentouri, M., Alexis, M., 2012. Growth performance, feed utilization and non-specific immune response of gilthead sea bream (*Sparus aurata* L.) fed graded levels of a bioprocessed soybean meal. Aquaculture 364, 74–81.
Krogdahl, A., Hemre, G.I., Mommsen, T.P., 2005. Carbohydrates in fish nutrition: digestion and absorption in postlarval stages. Aquac. Nutr. 11, 103–122.
Lall, S.P., Kaushik, S.J., Lebail, P.Y., Keith, R., Anderson, J.S., Plisetskaya, E., 1994. Quantitative arginine requirement of Atlantic Salmon (*Salmo salar*) reared in sea water. Aquaculture 124, 13–25.
Lin, H., Tan, X., Zhou, C., Niu, J., Xia, D., Huang, Z., Wang, J., Wang, Y., 2015. Effect of dietary arginine levels on the growth performance, feed utilization, non-specific immune response and disease resistance of juvenile golden pompano *Trachinotus ovatus*. Aquaculture 437, 382–389.
Luo, Z., Liu, Y.J., Mai, K.S., Tian, L.X., 2004. Advance in researches on arginine requirement for fish: a review. J. Fish. China 28, 450–459.
Luo, Z., Liu, Y.J., Mai, K.S., Tian, L.X., Tan, X.Y., Yang, H.J., 2007. Effects of dietary arginine levels on growth performance and body composition of juvenile grouper *Epinephelus coioides*. J. Appl. Ichthyol. 23, 252–257.
Martinez-Montano, E., Pena, E., Viana, M.T., 2013. Intestinal absorption of amino acids in the Pacific bluefin tuna (*Thunnus orientalis*): in vitro lysine-arginine interaction using the everted intestine system. Fish Physiol. Biochem. 39, 325–334.
McCord, J.M., Fridovich, I., 1969. Superoxide dismutase: an enzymic function for erythrocuprein. J. Biol. Chem. 244, 6049–6055.
Mommsen, T.P., Moon, T.W., Plisetskaya, E.M., 2001. Effects of arginine on pancreatic hormones and hepatic metabolism in rainbow trout. Physiol. Biochem. Zool. 74, 668–678.
Morales, A.E., Garciarejon, L., Delahiguera, M., 1990. Influence of handling and/or anesthesia on tress response in Rainbow-trout — effects on liver primary metabolism. Comp. Biochem. Physiol A 95, 87–93.
Morales, A.E., Perez-Jimenez, A., Hidalgo, M.C., Abellan, E., Cardenete, G., 2004. Oxidative stress and antioxidant defenses after prolonged starvation in *Dentex dentex* liver. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 139, 153–161.
Ngamsnae, P., De Silva, S.S., Gunasekera, R.M., 1999. Arginine and phenylalanine requirement of juvenile silver perch *Bidyanus bidyanus* and validation of the use of body amino acid composition for estimating individual amino acid requirements. Aquac. Nutr. 5, 173–180.
NRC, N.R.C., 2011. Nutrient Requirements of Fish and Shrimp. The National Academies Press, Washigton, DC.
Oliva-Teles, A., 2012. Nutrition and health of aquaculture fish. J. Fish Dis. 35, 83–108.
Oliva-Teles, A., Enes, P., Peres, H., 2015. Replacing fishmeal and fish oil in industrial aquafeeds for carnivorous species. In: Davies, A. (Ed.), Feed and Feeding Practices in Aquaculture. Woodhead Publishing Series in Food Science, Technology and Nutrition. Woodhead Publishing, Oxford, pp. 203–233.

Pan, M., Meng, Q., Choudry, H.A., Karinch, A.M., Lin, C., Souba, W.W., 2004. Stimulation of intestinal glutamine absorption in chronic metabolic acidosis. Surgery 136, 127–134.
Peres, H., Oliva-Teles, A., 2005. The effect of dietary protein replacement by crystalline amino acid on growth and nitrogen utilization of turbot *Scophthalmus maximus* juveniles. Aquaculture 250, 755–764.
Peres, H., Oliva-Teles, A., 2006. Effect of the dietary essential to non-essential amino acid ratio on growth, feed utilization and nitrogen metabolism of European sea bass (*Dicentrarchus labrax*). Aquaculture 256, 395–402.
Peres, H., Oliva-Teles, A., 2007. Effect of the dietary essential amino acid pattern on growth, feed utilization and nitrogen metabolism of European sea bass (*Dicentrarchus labrax*). Aquaculture 267, 119–128.
Peres, H., Oliva-Teles, A., 2008. Lysine requirement and efficiency of lysine utilization in turbot (*Scophthalmus maximus*) juveniles. Aquaculture 275, 283–290.
Peres, H., Oliva-Teles, A., 2009. The optimum dietary essential amino acid profile for gilthead seabream (*Sparus aurata*) juveniles. Aquaculture 296, 81–86.
Plisetskaya, E.M., Buchelli-Narvaez, L.I., Hardy, R.W., Dickhoff, W.W., 1991. Effects of injected and dietary arginine on plasma insulin levels and growth of pacific salmon and rainbow trout. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 98, 165.
Pohlenz, C., Gatlin Iii, D.M., 2014. Interrelationships between fish nutrition and health. Aquaculture 431, 111–117.
Pohlenz, C., Buentello, A., Miller, T., Small, B.C., MacKenzie, D.S., Gatlin, D.M., 2013. Effects of dietary arginine on endocrine growth factors of channel catfish, *Ictalurus punctatus*. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 166, 215–221.
Pohlenz, C., Buentello, A., Helland, S.J., Gatlin, D.M., 2014. Effects of dietary arginine supplementation on growth, protein optimization and innate immune response of channel catfish *Ictalurus punctatus* (Rafinesque 1818). Aquac. Res. 45, 491–500.
Ren, M., Liao, Y., Xie, J., Liu, B., Zhou, Q., Ge, X., Cui, H., Pan, L., Chen, R., 2013. Dietary arginine requirement of juvenile blunt snout bream, *Megalobrama amblycephala*. Aquaculture 414–415, 229–234.
Roehrig, K.L., Allred, J.B., 1974. Direct enzymatic procedure for the determination of liver glycogen. Anal. Biochem. 58, 414–421.
Sala-Rabanal, M., Gallardo, M.A., Sánchez, J., Planas, J.M., 2004. Na-dependent-glucose transport by intestinal brush border membrane vesicles from gilthead sea bream (*Sparus aurata*). J. Membr. Biol. 201, 85–96.
Salloum, R.M., Souba, W.W., Fernandez, A., Stevens, B.R., 1990. Dietary modulation of small intestinal glutamine transport in intestinal brush border membrane vesicles of rats. J. Surg. Res. 48, 635–638.
Santiago, C.B., Lovell, R.T., 1988. Amino acid requirements for growth of Nile tilapia. J. Nutr. 118, 1540–1546.
Santigosa, E., Garcia-Meilan, I., Valentin, J.M., Perez-Sanchez, J., Medale, F., Kaushik, S., Gallardo, M.A., 2011. Modifications of intestinal nutrient absorption in response to dietary fish meal replacement by plant protein sources in sea bream (*Sparus aurata*) and rainbow trout (*Onchorynchus mykiss*). Aquaculture 317, 146–154.
Shearer, K.D., 2000. Experimental design, statistical analysis and modelling of dietary nutrient requirement studies for fish: a critical review. Aquac. Nutr. 6, 91–102.
Singh, S., Khan, M.A., 2007. Dietary arginine requirement of fingerling hybrid Clarias (*Clarias gariepinus* × *Clarias macrocephalus*). Aquac. Res. 38, 17–25.
Sink, T.D., Lochmann, R.T., 2007. Insulin response of largemouth bass to glucose, amino acid, and diet stimulation. N. Am. J. Aquac. 69, 429–434.
Sitja-Bobadilla, A., Pena-Llopis, S., Gomez-Requeni, P., Medale, F., Kaushik, S., Perez-Sanchez, J., 2005. Effect of fish meal replacement by plant protein sources on non-specific defense mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). Aquaculture 249, 387–400.
Stein, E.D., Chang, S.D., Diamond, J.M., 1987. Comparison of different dietary amino acids as inducers of intestinal amino acid transport. Am. J. Phys. 252, 626–635.
Tibaldi, E., Kaushik, S.J., 2005. Amino acid requirements of Mediterranean fish species. Cah. Options Mediterr. 63, 59–65.
Tibaldi, E., Tulli, F., Lanari, D., 1994. Arginine requirement and effect of different dietary arginine and lysine levels for fingerling sea bass (*Dicentrarchus labrax*). Aquaculture 127, 207–218.
Trenzado, C., Hidalgo, M.C., Garcia-Gallego, M., Morales, A.E., Furne, M., Domezain, A., Domezain, J., Sanz, A., 2006. Antioxidant enzymes and lipid peroxidation in sturgeon *Acipenser naccarii* and trout *Oncorhynchus mykiss*. A comparative study. Aquaculture 254, 758–767.
Tulli, F., Vachot, C., Tibaldi, E., Fournier, V., Kaushik, S.J., 2007. Contribution of dietary arginine to nitrogen utilisation and excretion in juvenile sea bass (*Dicentrarchus labrax*) fed diets differing in protein source. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 147, 179–188.
Van Nguyen, M., Rønnestad, I., Buttle, L., Van Lai, H., Espe, M., 2014. Imbalanced lysine to arginine ratios reduced performance in juvenile cobia (*Rachycentron canadum*) fed high plant protein diets. Aquac. Nutr. 20, 25–35.
Vega-Rubín de Celis, S., Rojas, P., Gómez-Requeni, P., Albalat, A., Gutiérrez, J., Médale, F., Kaushik, S.J., Navarro, I., Pérez-Sánchez, J., 2004. Nutritional assessment of somatolactin function in gilthead sea bream (*Sparus aurata*): concurrent changes in somatotropic axis and pancreatic hormones. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 138, 533–542.
Wang, B., Liu, Y., Feng, L., Jiang, W.D., Kuang, S.Y., Jiang, J., Li, S.H., Tang, L., Zhou, X.Q., 2015. Effects of dietary arginine supplementation on growth performance, flesh quality, muscle antioxidant capacity and antioxidant-related signalling molecule expression in young grass carp (*Ctenopharyngodon idella*). Food Chem. 167, 91–99.
Watson, A.M., Barrows, T.F., Place, A.R., 2013. Taurine supplemented plant protein based diets with alternative lipid sources for juvenile gilthead sea bream, *Sparus aurata*. J. Int. Fish. Aquacult. 4, 59–66.
Weiser, M.M., 1973. Intestinal epithelial cell surface membrane glycoprotein synthesis. An indicator of cellular differentiation. J. Biol. Chem. 248, 2536–2546.
Wu, G., 2013. Protein digestion and absorption of peptides and amino acids. In: Wu, G. (Ed.), Amino Acids. CRC Press, Boca Raton, pp. 33–62.
Wu, G., Morris, S.M., 1998. Arginine metabolism: nitric oxide and beyond. Biochem. J. 336, 1–17.
Wu, G., Bazer, F., Burghardt, R., Johnson, G., Kim, S., Knabe, D., Li, P., Li, X., McKnight, J., Satterfield, M., Spencer, T., 2011. Proline and hydroxyproline metabolism: implications for animal and human nutrition. Amino Acids 40, 1053–1063.
Yue, Y., Zou, Z., Zhu, J., Li, D., Xiao, W., Han, J., Yang, H., 2015. Effects of dietary arginine on growth performance, feed utilization, haematological parameters and non-specific immune responses of juvenile Nile tilapia (*Oreochromis niloticus* L.). Aquac. Res. 46, 1801–1809.
Zhou, F., Xiong, W., Xiao, J.X., Shao, Q.J., Bergo, O.N., Hua, Y., Chai, X.J., 2010. Optimum arginine requirement of juvenile black sea bream, *Sparus macrocephalus*. Aquac. Res. 41, e418–e430.
Zhou, F., Shao, Q.J., Xiao, J.X., Peng, X., Ngandzali, B.O., Sun, Z., Ng, W.K., 2011. Effects of dietary arginine and lysine levels on growth performance, nutrient utilization and tissue biochemical profile of black sea bream, *Acanthopagrus schlegelii*, fingerlings. Aquaculture 319, 72–80.
Zhou, F., Zhou, J., Shao, Q., Wang, Y., Li, H., Gao, S., Hua, Y., 2012a. Effects of arginine-deficient and replete diets on growth performance, digestive enzyme activities and genes expression of black sea bream, *Acanthopagrus schlegelii*, juveniles. J. World Aquacult. Soc. 43, 828–839.
Zhou, Q.-C., Zeng, W.-P., Wang, H.-L., Xie, F.-J., Tuo, W., Zheng, C.-Q., 2012b. Dietary arginine requirement of juvenile yellow grouper *Epinephelus awoara*. Aquaculture 350–353, 175–182.
Zhou, Q., Jin, M., Elmada, Z.C., Liang, X., Mai, K., 2015. Growth, immune response and resistance to *Aeromonas hydrophila* of juvenile yellow catfish, *Pelteobagrus fulvidraco*, fed diets with different arginine levels. Aquaculture 437, 84–91.

Chapter 4

Effects of dietary methionine and taurine supplementation to low-fish meal diets on growth performance and oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles

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Effects of dietary methionine and taurine supplementation to low-fish meal diets on growth performance and oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles

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ABSTRACT

This study aimed to evaluate the effects of dietary methionine (Met) and taurine (Tau) supplementation on growth performance, feed utilization, and oxidative status of European sea bass juveniles. For that purpose, a 12-week growth trial was performed to test four isolipidic (18%) and isonitrogenous (6.7%) practical diets, containing 82% of the protein from plant origin and 18% from fish meal. Diets were formulated to have a Met level below (LMet) or above (HMet) requirements and were supplemented or not with 1% Tau (LMetTau and HMetTau). No dietary Met or Tau effects were observed on growth, feed utilization, and whole-body composition. Increasing dietary Met level led to increased catalase (CAT) and glutathione peroxidase (GPX) activities in the liver and total (tGSH) and reduced (GSH) glutathione in the intestine, while decreasing glutathione reductase (GR) and GPX activities in the intestine. Most of these effects were only observed in fish fed diets not supplemented with Tau. Dietary Tau supplementation decreased intestinal tGSH, GSH and oxidised (GSSH) glutathione content, the activity of GPX both in liver and intestine, and glucose-6-phosphate dehydrogenase (G6PDH) activity in the liver. Dietary Tau supplementation also affected hepatic CAT and intestinal GR activities, but effects were dependent of the dietary Met level. Overall, European sea bass seems to cope well with a plant-protein rich diet without Met or Tau supplementation. Both dietary Met and Tau were shown to modulate fish antioxidant response but without altering the oxidative damage level.

1. Introduction

The increased demand and cost of fishmeal (FM) and the increased awareness of the industry for the need to improve aquaculture sustainability led to significant progress on reducing aquafeeds dependency on finite marine-harvested resources (Tacon and Hasan, 2011). Plant feedstuffs have been used to replace FM due to its more constant availability and composition and lower costs, despite presenting amino acid (AA) imbalances, antinutritional factors, and lower protein content, digestibility and palatability than FM (Gatlin et al., 2007; Hardy, 2010). Nonetheless, in most intensively reared fish, a good proportion of the dietary FM may be replaced by plant protein sources (Oliva-Teles et al., 2015). Higher replacement levels usually require dietary supplementation with limiting AA, mainly methionine (Met) and lysine (Lys), so that growth rates, feed efficiency, and survival are not compromised (Kaushik et al., 2004; Kaushik et al., 1995; Li et al., 2009; Medale and Kaushik, 2009).

Besides being required for protein synthesis Met is also a methyl donor for several methylation reactions, including DNA, and a precursor of polyamines (spermine and spermidine), L-carnitine, and cysteine (Cys) (Espe et al., 2011; Finkelstein, 2000; Schuhmacher and Gropp, 1998). Moreover, the oxidation of Met residues via the methionine sulfoxide reductase system also acts as scavenger of reactive oxygen species (ROS), thus protecting cells from oxidative stress (Feng

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et al., 2011; Métayer et al., 2008; Weissbach et al., 2005).

The conditionally indispensable amino acid (CIAA) Cys is the main limiting AA for glutathione (GSH) synthesis, an important antioxidant molecule that acts as cofactor of glutathione peroxidase (GPX) on ROS reduction (Lu, 2000; Wu et al., 2004). Being a Cys precursor, Met can indirectly affect GSH generation with repercussions on cells oxidative status (Li et al., 2007; Métayer et al., 2008; Wu et al., 2004). In fact, previous observations in mammals revealed that an increase of oxidative stress, with consequent reduction of GSH, led to an enhancement of Met transsulfuration in order to meet Cys demand for GSH synthesis (Finkelstein, 2000).

Taurine (Tau), which also acts indirectly as antioxidant, is also synthesized from Cys through several enzymatic reactions, with cysteine sulfinate decarboxylase (CSD) appearing to be the rate-limiting enzyme in this process (Jacobsen and Smith, 1968; Lu, 2000). The ability of fish to synthetize Tau seems to increase with fish development and to vary between species, with accumulating evidences indicating that several marine finfish species are unable to or have limited capacity to synthesize Tau (Kim et al., 2005; Salze and Davis, 2015; Yokoyama et al., 2001), which explains why Tau is now considered a CIAA (Salze and Davis, 2015) or even an IAA in some fish species (Goto et al., 2001; Kim et al., 2005).

Tau is an amino sulfonic acid, which is not incorporated into proteins, but is an abundant free-AA in animal tissues. Likewise, Tau is found in relatively high concentrations in FM and especially in soluble fish protein concentrate, but is almost negligible in plant feedstuffs, with for instance soy beans not containing detectable levels of Tau (Spitze et al., 2003). Consequently, plant feedstuffs based diets for marine fish may need to be supplemented with Tau to guarantee that fish growth performance, survival, and disease resistance are not compromised (Goto et al., 2001; Kim et al., 2008; Matsunari et al., 2008; Oliva-Teles, 2012; Park et al., 2002; Takagi et al., 2010, 2011).

Taurine is known to play an important role in a wide variety of physiological functions, including cell membrane stabilization, modulation of calcium levels, anti-oxidation, anti-inflammation, osmoregulation and bile acid conjugation (Huxtable, 1992; Salze and Davis, 2015). In some marine fish fed a low FM feeds, a nutritional disease known as “green liver syndrome” has been reported, resulting from both a decreased excretion of Tau-conjugated bile pigment and an overproduction of hemolytic biliverdin (Sakai et al., 1990; Takagi et al., 2006; Takagi et al., 2005). In such cases, a dietay Tau supplementation has been found to be beneficial. In another species such as the totoaba (*Totoaba macdonaldi*), supplementation of 1% Tau to a diet with 60% FM replaced by soy protein concentrate was shown to restore lipid peroxidation levels and to increase catalase activity and the activity of key enzymes of the intermediary metabolism to the levels observed in fish fed the FM control diet (Bañuelos-Vargas et al., 2014).

European sea bass, a major marine finfish species of interest for the European aquaculture, was previously shown to grow well on a diet with an almost total replacement of dietary FM protein (95%) by a plant protein mixture supplemented with Lys but without Tau supplementation (Kaushik et al., 2004). Even though in Martinez et al. (2004) European sea bass fry were reported to have a dietary Tau requirement of 0.2%, in other fish species the ability to synthesize Tau has been shown to increase with the development stage, associated with the progressive increment of CSD activity (Kim et al., 2005; Yokoyama et al., 2001). In this sense it seems plausible to consider that European sea bass juveniles must at least have some Tau biosynthesis capacity.

In the context of developing low-FM feeds, understanding the linkage between Met, Cys and Tau, and their physiological roles, especially at the oxidative status level, deserves our attention. Therefore, the present study aimed at evaluating the effect of Met and Tau supplementation to a practical plant-based diet on growth performance, body composition, and oxidative status in juvenile European sea bass, a major marine finfish species of interest for the European aquaculture.

Table 1
Ingredient composition and proximate analysis of the experimental diets.

	LMet	LMetTau	HMet	HMetTau
Ingredients (% dry weight)				
Fish meal[a]	8.0	8.0	8.0	8.0
Soybean meal[b]	22.6	23.2	22.4	22.4
Wheat meal[c]	20.3	19.8	20.3	20.0
Corn gluten meal[d]	15.0	15.0	15.0	15.0
Wheat gluten meal[e]	5.0	5.0	5.0	5.0
Fish oil	14.8	14.8	14.8	14.8
Pea protein concentrate[f]	4.0	2.9	4.0	3.2
CPSP[g]	2.0	2.0	2.0	2.0
Dibasic calcium phosphate	3.8	3.8	3.8	3.8
Vitamins premix[h]	1.0	1.0	1.0	1.0
Choline chloride (50%)	0.5	0.5	0.5	0.5
Minerals premix[i]	1.0	1.0	1.0	1.0
Binder[j]	1.0	1.0	1.0	1.0
Agar	1.0	1.0	1.0	1.0
L-Methionine[k]	–	–	0.26	0.26
Taurine[k]	–	1.0	–	1.0
Proximate analysis (% dry weight)				
Dry matter (%)	90.5	93.0	90.6	92.1
Protein	42.4	43.3	43.4	43.6
Crude fat	18.0	17.8	17.6	17.8
Ash	8.9	9.1	9.1	9.2
Gross energy (kJ g^{-1})	22.5	22.7	22.4	22.7

[a] Pesquera Centinela, Steam Dried LT, Chile (CP: 71.0%; CL 11.3%). Sorgal, S.A. Ovar, Portugal.
[b] Soybean meal (CP: 56.4%; CL:2.5%), Sorgal, S.A. Ovar, Portugal.
[c] Wheat meal (CP: 11.5%; CL: 3.2%), Sorgal, S.A. Ovar, Portugal.
[d] Corn gluten meal (CP: 80.1%; CL: 4.1%), Sorgal, S.A. Ovar, Portugal.
[e] Wheat gluten meal CP: 84.8%; GL: 1.7%), Sorgal, S.A. Ovar, Portugal.
[f] Pea protein concentrate (CP: 84.0%; GL: 2.7%), Cosucra, Belgium.
[g] Soluble fish protein concentrate, Sopropêche, France (CP: 80.4% DM; GL: 15.7% DM).
[h] Vitamins (mg kg^{-1} diet): retinol, 18,000 (IU kg^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.
[i] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet).
[j] Aquacube. Agil, UK.
[k] Feed-grade amino acids, Sorgal, S.A. Ovar, Portugal.

2. Material and methods

2.1. Diets

Four practical diets were formulated to be isolipidic (18%) and isonitrogenous (6.7%), and to contain 18% protein from fishmeal (FM) and 82% protein provided by a mixture of plant feedstuffs. One diet (LMet diet) included Met below and another (HMet diet) above the established Met requirement for European sea bass (Tulli et al., 2010). Two other diets were formulated identical to the previous ones, but supplemented with 1% L-Tau (diets LMetTau and HMetTau, respectively). Care was taken to guarantee that all other dietary AA met the requirements for the species (Kaushik, 1998). Dietary ingredients were finely ground, well mixed, and dry pelleted through a 2.0 mm die in a pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA). Pellets were dried in an oven at 50 °C for 24 h and then stored in airtight bags until use. Ingredients and proximate composition of the experimental diets are presented in Table 1, and the AA composition of the experimental diets is presented in Table 2.

2.2. Growth trial

This experiment was directed by trained scientists (following FELASA category C recommendations) and was conducted according to the European Union Directive 2010/63/EU on the use of animals for

Table 2
Determined amino acid composition (% Diet) of the experimental diets.

	LMet	LMetTau	HMet	HMetTau
Methionine	0.74	0.75	0.96	0.98
Arginine	2.77	2.84	2.83	2.78
Histidine	0.98	1.02	1.00	1.00
Isoleucine	1.84	1.92	1.76	1.83
Leucine	4.08	4.31	3.93	4.03
Lysine	2.22	2.30	2.34	2.33
Threonine	1.63	1.67	1.58	1.58
Valine	2.19	1.89	1.97	2.01
Phenylalanine	2.46	2.67	2.64	2.61
Taurine	0.14	1.24	0.13	1.24
Tyrosine	1.67	1.68	1.77	1.70
Alanine	2.73	2.82	2.66	2.74
Aspartic acid	3.83	3.87	3.74	3.83
Glutamic acid	6.24	6.30	6.26	6.11
Glycine	3.12	2.99	2.84	2.91
Serine	2.22	2.29	2.34	2.27
Proline	2.24	2.41	2.15	2.37
Cysteine	0.59	0.65	0.62	0.65

scientific purposes. The growth trial was performed at the Marine Zoology Station, University of Porto, with European sea bass juveniles provided by IPMA/CRIPSul, Olhão, Portugal. Prior to the beginning of the trial fish were acclimated for 15 days to the experimental system and fed a commercial diet (Aquagold 3 mm, Aquasoja, Soja de Portugal, S.A). Thereafter, 12 groups of 14 fish with 6.9 ± 1.5 g mean body weight were randomly distributed into 12 cylindrical fibreglass tanks of 100 L capacity within a thermo-regulated semi-recirculation water system supplied with filtered seawater at a flow rate of 2.5–3.0 L min^{-1} and the experimental diets randomly assigned to triplicate groups of fish. During the trial water temperature was kept constant at 23.0 ± 1.0 °C, salinity averaged 35 ± 1‰, oxygen averaged 7–7.5 mg L^{-1}, ammonia and nitrite levels were kept around zero mg L^{-1} and a photoperiod of 12 h light and 12 h dark was adopted. Fish were hand-fed to apparent visual satiation twice a day, 6 days a week, during 85 days. Utmost care was taken that all feed supplied was consumed, and feed intake and mortality were daily recorded.

2.3. Sampling

Twelve fish from the initial batch were randomly sampled, euthanized by a lethal dose of anaesthesia (0.3 mg/L of ethylene glycol monophenyl ether) and pooled for whole-body composition analysis. At the end of the trial, 4 h after the morning meal, six fish per tank were euthanized by severing the spinal cord, and whole-fish, liver, and viscera weights were recorded for hepatosomatic index (HSI) and visceral index (VI) calculations. After sampling, liver and intestine (clear of adherent cells) from those fish were rapidly frozen in liquid nitrogen and then stored at − 80 °C until determination of enzyme activities. The carcass of three fish (without the liver) per tank were used for whole-body composition analysis.

2.4. Analytical methods

2.4.1. Chemical composition

Analysis of the experimental diets and whole-fish were done as follows: dry matter, by drying samples in an oven at 105 °C until constant weight; ash, by incineration in a muffle furnace at 450 °C for 16 h; crude protein content (N × 6.25) by the Kjeldahl method, after acid digestion using Kjeltec digestion and distillation units (Tecator Systems, Höganäs, Sweden; models 1015 and 1026, respectively); crude lipid, by petroleum ether extraction (SoxTec HT System); and gross energy by direct combustion of samples in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261).

Dietary samples for AA analysis were hydrolysed for 23 h with 6 N hydrochloric acid at 112 °C under N_2 atmosphere. Samples were then derivatized with phenylisothiocyanate (PITC) reagent before separation by gradient exchange chromatography (Waters auto sample model 717 plus; Waters binary pump model 1525; Waters dual absorbance detector model 2487), according to the Pico-Tag method as described by Cohen et al. (1989). Chromatographic peaks were identified, integrated, and quantified with a Waters Breeze software package by comparing to known protein hydrolysate AA standards (Pierce NC10180). Norleucine was used as internal standard. Tryptophan was determined according to Spies (1967).

2.4.2. Enzyme activities

Samples of liver and intestine (n = 9 per treatment) were diluted at 1:9 and 1:4, respectively, and homogenized at pH 7.8 in ice-cold 100 mM Tris–HCl buffer containing 0.1 mM EDTA and 0.1% (v/v) Triton X-100. All procedures were performed on ice. Homogenates were centrifuged at 30,000 × g for 30 min at 4 °C and the resultant supernatants were separated in aliquots and stored at − 80 °C for further enzyme assays. All enzyme activities were measured at 37 °C in a Multiskan GO microplate reader (Model5111 9200; Thermo Scientific, Nanjing, China).

Oxidative stress enzymes were analysed as follows: superoxide dismutase (SOD; EC 1.15.1.1) activity was measured at 550 nm by the ferricytochrome C method using xanthine/xanthine oxidase as source of superoxide radicals (McCord and Fridovich, 1969); catalase (CAT; EC 1.11.1.6) activity was determined according to Aebi (1984) by measuring the decrease of hydrogen peroxide concentration at 240 nm; glutathione reductase (GR; EC 1.6.4.2) activity was determined at 340 nm by measuring the oxidation of NADPH as described by Morales et al. (1990); glutathione peroxidase (GPX; EC 1.11.1.9) activity was assayed as described by Flohé and Günzler (1984); the oxidised glutathione (GSSG) generated by GPX was reduced by GR and the NADPH consumption rate was monitored at 340 nm. Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity was assayed as described by Morales et al. (1990) by monitoring the changes in absorbance of NADP at 340 nm. Protein concentration was determined according to Bradford (1976), using a commercial kit (Sigma protein Kit, cod. B6916) and bovine serum albumin as standard.

For SOD, one unit of enzyme activity was defined as the amount of enzyme necessary to produce 50% inhibition of the ferricytochrome C reduction rate. All other enzyme activities were expressed as units (CAT) or milliunits (G6PDH, GPX and GR) per milligram of hepatic soluble protein (specific activity). One unit of enzyme activity was defined as the amount of enzyme required to transform 1 μmol of substrate per minute under the assay conditions.

2.4.3. Lipid peroxidation

Hepatic and intestinal concentrations of thiobarbituric acid reacting substances were determined as marker of lipid peroxidation (LPO) following the methodology described by Buege and Aust (1978).

2.4.4. Total and oxidised glutathione

A portion of liver and intestine was homogenized (1:9 and 1:5, respectively) in ice-cold solution containing 1.3% 5-sulfosalicylic acid (w/v) and 10 mM HCl, and the whole procedures were undertaken on ice to avoid glutathione oxidation. Homogenates were centrifuged at 14,000g for 10 min at 4 °C and the resulting supernatants stored at − 80 °C. Total glutathione (tGSH) and oxidised glutathione (GSSG) were measured following the methods described by Griffith (1980) and Vandeputte et al. (1994) with some modifications (Perez-Jimenez et al., 2012). Standard curves of reduced glutathione (GSH) and GSSG were used for tGSH and GSSG calculations, respectively. GSH level was calculated by subtracting GSSG from tGSH values. Results are expressed as nmol g^{-1} tissue. The oxidative stress index (OSI) was calculated as follows:

$$OSI = 100 \times (2 \times GSSG/tGSH)$$

2.5. Statistical analysis

Growth performance, feed utilization, and whole-body composition data were analysed by two-way ANOVA, with dietary Met and Tau as fixed factors. Oxidative stress related parameters were analysed for the dietary Met and Tau effects by two-way ANOVA for liver and intestine separately. Fish were used as statistical units ($n = 9$) after verifying the absence of a tank effect through a three-way nested ANOVAs, with Met and Tau as fixed factors and the tank as aleatory factor. In the two-way ANOVA, if an interaction was significant, dietary Met and Tau effects were analysed by one-way ANOVA for each Tau or Met levels, respectively. All data were checked for normal distribution and homogeneity of variances and normalised when appropriate. Significant differences among means ($P < 0.05$) were determined by the Tukey's multiple range test. Statistical analysis was performed using SPSS for Windows version 22 software package.

3. Results

All experimental diets were well accepted by the fish and during the trial mortality was very low and unaffected by dietary treatments (Table 3). Weight gain, daily growth index, feed intake, feed efficiency and nitrogen retention were not affected by dietary Met or Tau (Table 3). At the end of the trial, there were no differences among groups on whole-body composition, HSI, and VI, except for the ash content that was higher in fish fed the high Met diets (Table 4).

The activities of liver and intestine antioxidant enzymes are presented in Table 5. The liver presented substantially higher G6PDH activity than the intestine, while the intestine showed higher CAT, SOD, GR and GPX activities. In the liver, GR and SOD activities were not affected by diets, while dietary Tau supplementation decreased G6PDH and GPX activities. CAT activity was also decreased by Tau supplementation in high Met diets, but the inverse was observed for the low Met diets. High dietary Met increased GPX activity and this effect was counteracted by dietary Tau supplementation. In the intestine, diets did not affect G6PDH, CAT and SOD activities. High dietary Met and Tau decreased GR and GPX activities, although Tau had the opposite effect in the high Met diets.

Data on liver and intestine tGSH, GSH, GSSG and LPO contents and OSI are presented in Table 6. All parameters were higher in the liver than in the intestine except for LPO, which was higher in the intestine. In the liver, none of these parameters was affected by diet composition. In the intestine, OSI and LPO were also unaffected by diet composition. On the contrary, the intestinal tGSH and GSH contents increased with dietary Met, while dietary Tau decreased tGSH, GSH and GSSG content.

4. Discussion

European sea bass is known to accept and grow well with feeds containing high levels of plant ingredients (Oliva-Teles et al., 2015). In fact, it was already shown that it can cope with up to 95% fishmeal protein replacement by a mixture of plant feedstuffs provided that the dietary indispensable amino acids (IAA) profile is met (Kaushik et al., 2004). The present study corroborates these observations, as growth performance and feed/nitrogen utilization of fish fed an 8% FM diet (corresponding to 5.7% protein) were similar to that of previous studies in European sea bass fed high fishmeal diets (Enes et al., 2006; Moreira et al., 2008; Peres and Oliva-Teles, 1999, 2005; Tulli et al., 2010).

All fish as other animals are recognised to have a dietary need for both Met and total sulphur AA (TSAA; Met + Cys) (Wilson, 2002). For European sea bass, Met requirement for maximum growth was estimated to be 1.3% (35 g fish; Thebault et al., 1985) or 0.9% of the diet (Hidalgo et al., 1987), corresponding to a TSAA level of 1.9 to 2.2%, respectively. More recently, Tulli et al. (2010) estimated Met requirement to be 0.8–0.9% of the diet and TSAA requirement to be 1.2–1.3% of the diet (13 g European sea bass), and observed no depression of growth or N retention at the highest Met and TSAA levels tested (1.6% and 2.0% of the diet, respectively).

In the present study, although the Met level of the LMet diet (0.75% diet) was lower than the reported Met requirement for this species, growth and feed utilization was not affected, indicating that fish were able to overcome this apparent marginal Met deficiency. Feed intake between dietary treatments was also not affected, and given that one primary consequence of feeding fish a diet with IAA imbalances is the reduction in voluntary feed intake (de la Higuera, 2001), this result also attests for the absence of a severe IAA deficiency. These results could also indicate a higher efficiency of Met utilization in fish fed marginal deficient Met diets (Encarnação et al., 2004; Peres and Oliva-Teles, 2008; Rodehutscord et al., 1997). Furthermore, dietary Cys level may also have contributed to spare Met through the reduction of Met transsulfuration (Fukagawa, 2006). As Met is an irreversible precursor of Cys, and TSAA requirements can be met by Met alone or by a combination of Met and Cys (Baker, 2006), circa 40% to 60% of the dietary Met requirements, on a weight basis, can be spared by Cys, under adequate TSAA levels (Abidi and Khan, 2011; Farhat and Khan, 2014; Goff and Gatlin, 2004; Griffin et al., 1994; Harding et al., 1977; Nguyen and Davis, 2009; NRC, 2011; Wilson, 2002).

Table 3
Growth performance and feed utilization of European sea bass fed the experimental diets.

Diets	LMet	LMetTau	HMet	HMetTau	2-Way ANOVA		
					Met	Tau	Met ∗ Tau
Final body weight (g)	26.5 ± 1.1	25.9 ± 2.8	24.6 ± 3.5	28.4 ± 2.7	NS	NS	NS
Weight gain (g kg ABW^{-1} day^{-1})	13.8 ± 0.3	13.5 ± 0.9	13.1 ± 1.2	14.3 ± 0.7	NS	NS	NS
Feed Intake (g kg ABW^{-1} day^{-1})	18.9 ± 0.9	19.0 ± 0.8	19.2 ± 0.5	19.4 ± 0.5	NS	NS	NS
Feed efficiency[a]	0.73 ± 0.04	0.71 ± 0.07	0.69 ± 0.07	0.74 ± 0.05	NS	NS	NS
Daily growth index[b]	1.27 ± 0.05	1.23 ± 0.13	1.18 ± 0.17	1.34 ± 0.11	NS	NS	NS
Protein efficiency ratio[c]	1.7 ± 0.1	1.7 ± 0.2	1.6 ± 0.2	1.7 ± 0.1	NS	NS	NS
Nitrogen intake (g kg ABW^{-1} day^{-1})[d]	1.3 ± 0.06	1.3 ± 0.06	1.3 ± 0.03	1.4 ± 0.04	NS	NS	NS
Nitrogen retention (g kg ABW^{-1} day^{-1})[e]	0.37 ± 0.01	0.37 ± 0.03	0.36 ± 0.04	0.39 ± 0.03	NS	NS	NS
Mortality (%)	2.4 ± 4.1	4.8 ± 4.1	2.4 ± 4.1	2.4 ± 4.1	NS	NS	NS

Values presented as mean ± S.D. ($n = 3$). NS: non-significant ($P \geq 0.05$).
ABW: average body weight (initial body weight, IBW + final body weight, FBW)/2.
[a] Feed efficiency: wet weight gain/dry feed intake.
[b] Daily growth index: (FBW1/3 − IBW1/3)/time in days) × 100.
[c] Protein efficiency ratio: wet weight gain/crude protein intake.
[d] Nitrogen intake: (N intake × 1000) / (ABW x time in days).
[e] Nitrogen retention: (FBW × FBN) − (IBW × IBN) /(ABW × time in days); FBN and IBN: final and initial whole-body N content, respectively.

Table 4
Whole-body composition (% wet weight), hepatosomatic index (HSI) and visceral index (VI) of European sea bass fed the experimental diets.

Diets	Initial	LMet	LMetTau	HMet	HMetTau	2-Way ANOVA		
						Met	Tau	Met * Tau
Whole-body composition								
Dry matter	31	32 ± 0.7	32 ± 0.9	32 ± 0.8	32 ± 1.0	NS	NS	NS
Ash	6	4.6 ± 0.1	4.5 ± 0.1	4.8 ± 0.2	4.7 ± 0.2	*	NS	NS
Lipid	11	11 ± 0.7	11 ± 0.6	11 ± 1.5	11 ± 1.1	NS	NS	NS
Protein	17	17 ± 0.2	17 ± 0.4	17 ± 0.4	17 ± 0.4	NS	NS	NS
Indexes								
HSI	ND	1.3 ± 0.17	1.4 ± 0.22	1.5 ± 0.24	1.4 ± 0.22	NS	NS	NS
VI	ND	17 ± 2.2	15 ± 2.7	15 ± 2.7	15 ± 2.4	NS	NS	NS

Values presented as mean ± S.D. ($n = 3$). Two-way ANOVA: NS - non-significant ($P \geq 0.05$); *$P < 0.05$. ND: not determined.

Indeed, in the present study, even though Met level was possibly provided at sub-adequate levels in the LMet diet, TSAA was 1.36% of the diet, well above the estimated requirement for the species (TSAA 1.2–1.3% of the diet; Tulli et al. (2010)). This is a practical consequence of using plant feedstuffs in diet formulation, as the majority of plant feedstuffs have low Met:Cys ratios (NRC, 2011). In dose-response studies, dietary Met and TSAA requirements are usually estimated using diets devoid of Cys (Goff and Gatlin, 2004; Harding et al., 1977) or with low Cys levels, from 0.04 to 0.4% (Abidi and Khan, 2011; Farhat and Khan, 2014; Griffin et al., 1994; Nguyen and Davis, 2009; Tulli et al., 2010). Thus, a substantial amount of the established Met requirements may be driven for Cys synthesis (Abidi and Khan, 2011; Baker, 2006; Figueiredo-Silva et al., 2015). Consequently, in diets with adequate Cys level the real Met requirement may in fact be lower than the published values. With Met being the first limiting AA in several plant feedstuffs, re-establishing Met requirement in practical diets, which may have high Cys content, would be of major interest for European sea bass aquaculture industry.

Animal feedstuffs, including FM, are recognised as relatively rich in Tau compared to plant feedstuffs (Spitze et al., 2003). Thus, Tau would be of limited concern in FM-based diets. However, Tau supplementation to low fishmeal or all-plant diets was shown to enhance fish performance in some species (Koven et al., 2016; Li et al., 2016), while not in others (Kim et al., 2008).

In European sea bass fry, a 0.2% Tau supplementation of a FM-based diet (60% of the diet), including 15.6% soybean meal, there was an enhanced growth performance and feed efficiency (Martinez et al., 2004). In another study with the same species, 1% Tau supplementation of a low-FM diet (25% of the diet) did not improve growth and feed utilization (Feidantsis et al., 2014). As mentioned earlier, it was previously reported that juvenile European sea bass were able to cope with a low-FM diet (5% DM) without Tau supplementation (Kaushik et al., 2004). Likewise, in the present study dietary Tau supplementation did not induce any enhancement in growth or feed utilization of sea bass juveniles fed a diet with only 8% FM. The apparent contradictory results between fry and juveniles may be related to European sea bass capacity of endogenous Tau biosynthesis throughout its life stages. Indeed, previous studies suggest that the ability to synthesize Tau varies according to the development stage, due to the progressive increment of cysteine sulfinate decarboxylase (CSD) activity (Kim et al., 2005; Yokoyama et al., 2001).

Tau can be synthesized from Cys, and the activity of CSD may be the rate-limiting step of Tau biosynthesis from Cys and, therefore, the main determinant of the extent of endogenous Tau production in fish (Goto et al., 2003; Yokoyama et al., 2001). In fact, CSD activity was previously shown to have a wide variability among fish species and to lack in fast growing carnivorous such as yellowtail (*Seriola quinqueradiata*), and bluefin tuna (*Thunnus thynnus*) and skipjack tuna (*Katsuwonus pelamis*) (Yokoyama et al., 2001). In the current study dietary Tau supplementation did not improve European sea bass growth performance,

Table 5
Liver and intestine antioxidant enzymes activity levels of European sea bass fed the experimental diets.

Organ	Liver				Intestine			
Diets	LMet	LMetTau	HMet	HMetTau	LMet	LMetTau	HMet	HMetTau
G6PDH	318 ± 47	263 ± 49	295 ± 74	255 ± 53	2.1 ± 1.3	1.2 ± 0.8	1.0 ± 0.9	1.0 ± 0.7
CAT	177 ± $9^{a, A}$	213 ± 26^{B}	287 ± $15^{b, B}$	231 ± 30^{A}	348 ± 55	347 ± 23	314 ± 50	363 ± 56
GR	3.6 ± 1.1	3.7 ± 0.9	4.0 ± 0.9	2.9 ± 0.6	52 ± $2^{b, B}$	47 ± 52^{A}	43 ± $72^{a, A}$	51 ± 5^{B}
SOD	86 ± 18	83 ± 23	101 ± 27	77 ± 25	342 ± 70	317 ± 103	297 ± 38	280 ± 66
GPX	25 ± 5	17 ± 7	32 ± 8	25 ± 8	74 ± $15^{b, B}$	46 ± 8^{A}	46 ± 5^{a}	53 ± 7

Two-way[a] ANOVA	Liver			Intestine		
	Met	Tau	Met * Tau	Met	Tau	Met * Tau
G6PDH	NS	*	NS	NS	NS	NS
CAT	***	NS	***	NS	NS	NS
GR	NS	NS	NS	NS	NS	**
SOD	NS	NS	NS	NS	NS	NS
GPX	**	**	NS	**	**	***

Values presented as mean ± S.D. ($n = 9$). Enzyme activities expressed as mU mg protein^{-1} for glucose-6-phosphate dehydrogenase (G6PDH), glutathione reductase (GR) and glutathione peroxidase (GPX), and as U mg protein^{-1} for catalase (CAT) and superoxide dismutase (SOD). NS - non-significant ($P \geq 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
If interaction was significant, dietary Met and Tau effects were analysed by one-way ANOVA for each Tau and Met levels, respectively; means in the same line with different small or capital letters indicate significant differences ($P < 0.05$) for Met or Tau levels, respectively; means with no letters are not significantly different ($P > 0.05$).
[a] Two-way ANOVA on dietary Met and Tau effects.

Table 6
Liver and intestine total glutathione (tGSH), reduced glutathione (GSH), oxidised glutathione (GSSG), oxidative stress index (OSI) and lipid peroxidation (LPO) levels of European sea bass fed the experimental diets.

Organ	Liver				Intestine			
Diets	LMet	LMetTau	HMet	HMetTau	LMet	LMetTau	HMet	HMetTau
tGSH	1566 ± 355	1609 ± 235	1563 ± 355	1270 ± 262	811 ± 186	638 ± 216	1103 ± 251	795 ± 298
GSH	1391 ± 382	1439 ± 235	1389 ± 356	1103 ± 293	755 ± 177	596 ± 217	1039 ± 242	747 ± 289
GSSG	175 ± 53	170 ± 42	174 ± 47	167 ± 50	57 ± 11	42 ± 5	64 ± 14	47 ± 12
OSI	24 ± 11	21 ± 6	23 ± 7	28 ± 11	14 ± 2	14 ± 4	12 ± 2	13 ± 3
LPO	17 ± 2	16 ± 4	17 ± 3	19 ± 4	27 ± 3	26 ± 3	26 ± 3	26 ± 3

Two-way[a] ANOVA	Liver			Intestine		
	Met	Tau	Met*Tau	Met	Tau	Met*Tau
tGSH	NS	NS	NS	*	*	NS
GSH	NS	NS	NS	*	*	NS
GSSG	NS	NS	NS	NS	***	NS
OSI	NS	NS	NS	NS	NS	NS
LPO	NS	NS	NS	NS	NS	NS

Values presented as mean ± S.D. ($n = 9$). Lipid peroxidation (LPO) values expressed as nmols MDA g^{-1} tissue; total glutathione (tGSH), reduced glutathione (GSH) and oxidised glutathione (GSSG) as nmol g^{-1} tissue; and oxidative stress index (OSI) as percentage.
NS - non-significant ($P \geq 0.05$); * $P < 0.05$; *** $P < 0.001$.
[a] Two-way ANOVA on dietary Met and Tau effects.

suggesting that Tau biosynthetic pathway should present some activity in this species, an aspect which deserves further investigation.

Dietary Met and Tau supplementation also had no significant effects on HSI, VI, and whole-body composition, also suggesting that the LMet diet was not deficient in either Met or Tau. Similarly, Tulli et al. (2010) reported that European sea bass whole body N content was little affected by dietary Met level, while in common dentex (*Dentex dentex*) Tau was shown to have little effects on whole-body, muscle and liver proximate composition (Chatzifotis et al., 2008).

The intestine usually has a higher GPX and GR activities and GSSG content than the liver, while the liver shows higher CAT activity and tGSH and GSH contents, with similar SOD activity in both organs (Castro et al., 2015; Coutinho et al., 2016; Trenzado et al., 2006). We observe here that European sea bass show higher activity of all anti-oxidative stress enzymes and lower glutathione content in the intestine than in the liver, suggesting that the diets used induced some oxidative stress in the intestine. According to Castro et al. (2015), diet composition affects oxidative stress of European sea bass in a tissue-related manner and the intestine is strongly responsive to dietary oil/fatty acid source. In the study of Castro et al. (2015) diets were FM-based (64.5–86.5% FM) while in the present study diets only included 8% FM. The high oxidative-stress enzyme activity observed in the intestine may possibly be explained by the need to counteract the high oxidation potential induced by these more extreme diets. Indeed, in a previous study with European sea bass, fish fed a 70% plant protein diet presented higher LPO content and SOD and CAT activities and lower GPX activity in the liver than fish fed a fish meal based diet, indicating that high dietary plant protein increased superoxide anion production (Guerreiro et al., 2015).

As generally observed, intestine also presented higher LPO values than liver. Accordingly, 2-fold higher LPO values were previously observed in the intestine of European sea bass fed a FM-based diet (Castro et al., 2015). This is justified by the high exposure of intestine to oxidative damage, due to enterocytes high turnover rates (Castro et al., 2015; Coutinho et al., 2016).

Liver is the main GSH producer and storage organ (Lu, 2000; Ojopagogo et al., 2013) and supplies other tissues including the intestine (Wu et al., 2004). This function is related to the ability of liver to convert Met to Cys, a rate-limiting AA for GSH biosynthesis (Lu, 2000; Ojopagogo et al., 2013). In fish, dietary Met or methionine hydroxy analogue (MHA) led to an increase in serum (Xiao et al., 2012) and hepatic GSH (Feng et al., 2011; Fontagné-Dicharry et al., 2017). Interestingly, Keembiyehetty and Gatlin (1995) reported that in juvenile sunshine bass the increase in hepatic GSH was even higher with Met supplemented diets than when supplemented with GSH in equal-sulphur basis. Accordingly, in the present study dietary Met supplementation increased tGSH and GSH contents in the intestine, although such effect was not observed in the liver.

An increase in dietary Met also led to an increase in GPX and CAT activities in the liver, but only in the diets without Tau supplementation for the latter. In gilthead seabream, dietary Met supplementation also led to an increase in GPX, SOD and CAT activities (Perez-Jimenez et al., 2012) and in juvenile Jian carp (*Cyprinus carpio* var. Jian) optimum dietary MHA increased CAT, GPX, SOD, and GR activities in muscle, serum, hepatopancreas, and intestine (except for SOD in muscle) (Feng et al., 2011; Xiao et al., 2012). Such an induction of antioxidant enzymes activity by dietary Met is reportedly associated with the role of Met as a methyl donor for DNA methylation, similarly to what has been observed in rats (Feng et al., 2011; Seneviratne et al., 1999; Xiao et al., 2012).

In the intestine, the LMet diet promoted a generalised higher activity of antioxidant enzymes, with GPX and GR activities being significantly reduced with further Met and Tau supplementation. Such results reveal that the low dietary Met and Tau levels increased the intestine's susceptibility to oxidative damage, attesting for these AA involvement in anti-oxidative processes (Métayer et al., 2008; Salze and Davis, 2015).

In the liver, dietary Tau supplementation led to a decrease of GPX activity while in the intestine such effect was only noticed in the LMet diets. Tau also affected CAT activity in the liver, but results were dependent on the dietary Met level. Nonetheless, no differences in LPO values were noticed neither in liver or intestine. This balancing of CAT by dietary Tau, leading to a more intermediate activity, and the downregulation of GPX activity, agree with the previously mentioned Tau role in the reduction of oxidants production in the first place (Jong et al., 2012; Salze and Davis, 2015), thereby reducing the need for high antioxidant enzymes activities. It is interesting to note that while in the liver Tau supplementation to the HMet diet led to a decrease of CAT and GPX activities, the opposite occurred in the intestine for GPX and GR activities. Moreover, dietary Tau decreased tGSH, GSH and GSSG

contents in the intestine, which is contrary to the effects observed for the lower dietary Met level in GPX and GR activities, the two main enzymes that use GSH as subtract.

Dietary Tau supplementation was reported to increase glucose phosphorylation and hepatic G6PDH activity in totoaba (Bañuelos-Vargas et al., 2014), but the opposite was observed in the present study. G6PDH is the main supplier of reducing power (NADPH) for both lipogenesis and antioxidant response, being crucial for normal CAT functioning and GR activity, which is responsible for GSH regeneration from GSSG (Morales et al., 2004; Perez-Jimenez et al., 2012; Scott et al., 1991). Thus, this reduction of G6PDH activity is apparently contradictory to the observed CAT and GR activities. A significant decrease of G6PDH activity would potentially decrease CAT and GR activities, as well as GSH and GSSG contents in the liver, which did not occur.

Precise quantitative data on antioxidant effects of Tau in fish are scarce and the mechanisms underlying Tau antioxidant activity are yet to be fully understood (Hansen and Grunnet, 2013; Jong et al., 2012). It is however possible that Tau antioxidant action is also influenced by GSH, since Tau and GSH are reported to interplay in the mitochondria, regulating oxidation and possibly ROS generation (Hansen and Grunnet, 2013).

Despite the effects of Tau on the activity of some oxidative enzymes in liver and intestine, both OSI and LPO values were maintained, revealing that the cellular redox state and oxidative damage remained unchanged. In another study in European sea bass, LPO values were also unaffected by dietary Tau (Feidantsis et al., 2014), while in totoaba (Bañuelos-Vargas et al., 2014) and yellow catfish (*Pelteobagrus fulvidraco*) (Li et al., 2016) Tau decreased LPO values in the liver and serum, respectively.

Overall, results of the present study indicate that European sea bass juveniles perform well with a plant feedstuff based diet with a Met level 12% below the established requirement for the species and without Tau supplementation. Dietary Met and Tau supplementation to the diet modulate both hepatic and intestinal antioxidant response, but do not affect overall liver and intestine oxidative status.

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References

Abidi, S.F., Khan, M.A., 2011. Total sulphur amino acid requirement and cystine replacement value for fingerling rohu, *Labeo rohita*: effects on growth, nutrient retention and body composition. Aquac. Nutr. 17, e583–e594.
Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.
Baker, D.H., 2006. Comparative species utilization and toxicity of sulfur amino acids. J. Nutr. 136, 1670S–11675.
Bañuelos-Vargas, I., López, L.M., Pérez-Jiménez, A., Peres, H., 2014. Effect of fishmeal replacement by soy protein concentrate with taurine supplementation on hepatic intermediary metabolism and antioxidant status of totoaba juveniles (*Totoaba macdonaldi*). Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 170, 18–25.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Methods Enzymol. 52, 302–310.
Castro, C., Perez-Jimenez, A., Coutinho, F., Diaz-Rosales, P., Serra, C.A., Panserat, S., Corraze, G., Peres, H., Oliva-Teles, A., 2015. Dietary carbohydrate and lipid sources affect differently the oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles. Br. J. Nutr. 114, 1584–1593.
Chatzifotis, S., Polemitou, I., Divanach, P., Antonopouiou, E., 2008. Effect of dietary taurine supplementation on growth performance and bile salt activated lipase activity of common dentex, *Dentex dentex*, fed a fish meal/soy protein concentrate-based diet. Aquaculture 275, 201–208.
Cohen, S.A., Meys, M., Tarvin, T., 1989. The Pico-Tag method. A manual of advance techniques for amino acid analysis. Waters chromatography division, Milford, MA.
Coutinho, F., Castro, C., Rufino-Palomares, E., Ordonez-Grande, B., Gallardo, M.A., Oliva-Teles, A., Peres, H., 2016. Dietary glutamine supplementation effects on amino acid metabolism, intestinal nutrient absorption capacity and antioxidant response of gilthead sea bream (*Sparus aurata*) juveniles. Comp. Biochem. Physiol. A Comp. Physiol. 191, 9–17.
Encarnação, P., de Lange, C., Rodehutscord, M., Hoehler, D., Bureau, W., Bureau, D.P., 2004. Diet digestible energy content affects lysine utilization, but not dietary lysine requirements of rainbow trout (*Oncorhynchus mykiss*) for maximum growth. Aquaculture 235, 569–586.
Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2006. Rapid metabolic adaptation in European sea bass (*Dicentrarchus labrax*) juveniles fed different carbohydrate sources after heat shock stress. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 145, 73–81.
Espe, M., Liaset, B., Hevroy, E.M., El-Mowafi, A., 2011. DL-methionine enrichment in diets fed to Atlantic salmon increases apparent digestibility. Aquac. Res. 42, 1123–1130.
Farhat, Khan, M.A., 2014. Total sulfur amino acid requirement and cystine replacement value for fingerling stinging catfish, *Heteropneustes fossilis* (Bloch). Aquaculture 426, 270–281.
Feidantsis, K., Kaitetzidou, E., Mavrogiannis, N., Michaelidis, B., Kotzamanis, Y., Antonopoulou, E., 2014. Effect of taurine-enriched diets on the Hsp expression, MAPK activation and the antioxidant defence of the European sea bass (*Dicentrarchus labrax*). Aquac. Nutr. 20, 431–442.
Feng, L., Xiao, W.W., Liu, Y., Jiang, J., Hu, K., Jiang, W.D., Li, S.H., Zhou, X.Q., 2011. Methionine hydroxy analogue prevents oxidative damage and improves antioxidant status of intestine and hepatopancreas for juvenile Jian carp (*Cyprinus carpio* Var. Jian). Aquac. Nutr. 17, 595–604.
Figueiredo-Silva, C., Lemme, A., Sangsue, D., Kiriratnikom, S., 2015. Effect of DL-methionine supplementation on the success of almost total replacement of fish meal with soybean meal in diets for hybrid tilapia (*Oreochromis niloticus* × *Oreochromis mossambicus*). Aquac. Nutr. 21, 234–241.
Finkelstein, J.D., 2000. Pathways and regulation of homocysteine metabolism in mammals. Semin. Thromb. Hemost. 26, 219–225.
Flohé, L., Günzler, W.A., 1984. Assay of glutathione peroxidase. Methods Enzymol. 105, 115–121.
Fontagné-Dicharry, S., Alami-Durante, H., Aragão, C., Kaushik, S.J., Geurden, I., 2017. Parental and early-feeding effects of dietary methionine in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 469, 16–27.
Fukagawa, N.K., 2006. Sparing of methionine requirements: evaluation of human data takes sulfur amino acids beyond protein. J. Nutr. 136, 1676S–1681S.
Gatlin, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W., Herman, E., Hu, G., Krogdahl, Å., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., Souza, J., Stone, D., Wilson, R., Wurtele, E., 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquac. Res. 38, 551–579.
Goff, J.B., Gatlin, D.M., 2004. Evaluation of different sulfur amino acid compounds in the diet of red drum, *Sciaenops ocellatus*, and sparing value of cystine for methionine. Aquaculture 241, 465–477.
Goto, T., Takagi, S., Ichiki, T., Sakai, T., Endo, M., Yoshida, T., Ukawa, M., Murata, H., 2001. Studies on the green liver in cultured red sea bream fed low level and non-fish meal diets: relationship between hepatic taurine and biliverdin levels. Fish. Sci. 67, 58–63.
Goto, T., Matsumoto, T., Murakami, S., Takagi, S., Hasumi, F., 2003. Conversion of cysteate into taurine in liver of fish. Fish. Sci. 69, 216–218.
Griffin, M.E., White, M.R., Brown, P.B., 1994. Total sulfur amino acid requirement and cysteine replacement value for juvenile hybrid striped bass (*Morone saxatilis* × *M. chrysops*). Comp. Biochem. Physiol. A Comp. Physiol. 108, 423–429.
Griffith, O.W., 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal. Biochem. 106, 207–212.
Guerreiro, I., Couto, A., Pérez-Jiménez, A., Oliva-Teles, A., Enes, P., 2015. Gut morphology and hepatic oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles fed plant feedstuffs or fishmeal-based diets supplemented with short-chain fructo-oligosaccharides and xylo-oligosaccharides. Br. J. Nutr. 114, 1975–1984.
Hansen, S.H., Grunnet, N., 2013. Taurine, glutathione and bioenergetics. Adv. Exp. Med. Biol. 776, 3–12.
Harding, D.E., Allen, O.W., Wilson, R.P., 1977. Sulfur amino acid requirement of channel catfish - L-methionine and L-Cystine. J. Nutr. 107, 2031–2035.
Hardy, R.W., 2010. Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. Aquac. Res. 41, 770–776.
Hidalgo, F., Alliot, E., Thebault, H., 1987. Methionine- and cystine-supplemented diets for juvenile sea bass (*Dicentrarchus labrax*). Aquaculture 64, 209–217.
de la Higuera, M., 2001. Effects of Nutritional Factors and Feed Characteristics on Feed Intake, Food Intake in Fish. pp. 250–268.
Huxtable, R.J., 1992. Phisiological actions of taurine. Physiol. Rev. 72, 101–163.
Jacobsen, J.G., Smith, L.H., 1968. Biochemistry and physiology of Taurine and Taurine derivatives. Physiol. Rev. 18.
Jong, C.J., Azuma, J., Schaffer, S., 2012. Mechanism underlying the antioxidant activity of taurine: prevention of mitochondrial oxidant production. Amino Acids 42, 2223–2232.
Kaushik, S.J., 1998. Whole body amino acid composition of European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and turbot (*Psetta maxima*) with an estimation of their IAA requirement pro files. Aquat. Living Resour. 11,

355–358.
Kaushik, S.J., Cravedi, J.P., Lalles, J.P., Sumpter, J., Fauconneau, B., Laroche, M., 1995. Partial or Total replacement of fish meal by soybean protein on growth, protein utilization, potential estrogenic or antigenic effects, Cholesterolemia and flesh quality in rainbow trout, *Oncorhynchus mykiss*. Aquaculture 133, 257–274.
Kaushik, S.J., Coves, D., Dutto, G., Blanc, D., 2004. Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus labrax*. Aquaculture 230, 391–404.
Keembiyehetty, C.N., Gatlin, D.M., 1995. Evaluation of different sulfur compounds in the diet of juvenile sunshine bass (*Morone chrysops* ♀ × *M. saxatilis* ♂). Comp. Biochem. Physiol. A Comp. Physiol. 112, 155–159.
Kim, S.-K., Takeuchi, T., Yokoyama, M., Murata, Y., Kaneniwa, M., Sakakura, Y., 2005. Effect of dietary taurine levels on growth and feeding behavior of juvenile Japanese flounder *Paralichthys olivaceus*. Aquaculture 250, 765–774.
Kim, S.K., Matsunari, H., Takeuchi, T., Yokoyama, M., Furuita, H., Murata, Y., Goto, T., 2008. Comparison of taurine biosynthesis ability between juveniles of Japanese flounder and common carp. Amino Acids 35, 161–168.
Koven, W., Peduel, A., Gada, M., Nixon, O., Ucko, M., 2016. Taurine improves the performance of white grouper juveniles (*Epinephelus aeneus*) fed a reduced fish meal diet. Aquaculture 460, 8–14.
Li, P., Yin, Y.L., Li, D., Kim, S.W., Wu, G.Y., 2007. Amino acids and immune function. Br. J. Nutr. 98, 237–252.
Li, P., Mai, K.S., Trushenski, J., Wu, G.Y., 2009. New developments in fish amino acid nutrition: towards functional and environmentally oriented aquafeeds. Amino Acids 37, 43–53.
Li, M., Lai, H., Li, Q., Gong, S., Wang, R., 2016. Effects of dietary taurine on growth, immunity and hyperammonemia in juvenile yellow catfish *Pelteobagrus fulvidraco* fed all-plant protein diets. Aquaculture 450, 349–355.
Lu, S.C., 2000. Regulation of glutathione synthesis. Curr. Top. Cell. Regul. 36, 95–116.
Martinez, J.B., Chatzifotis, S., Divanach, P., Takeuchi, T., 2004. Effect of dietary taurine supplementation on growth performance and feed selection of sea bass *Dicentrarchus labrax* fry fed with demand-feeders. Fish. Sci. 70, 74–79.
Matsunari, H., Yamamoto, T., Kim, S.K., Goto, T., Takeuchi, T., 2008. Optimum dietary taurine level in casein-based diet for juvenile red sea bream *Pagrus major*. Fish. Sci. 74, 347–353.
McCord, J.M., Fridovich, I., 1969. Superoxide dismutase: an enzymic function for erythrocuprein. J. Biol. Chem. 244, 6049–6055.
Medale, F., Kaushik, S., 2009. Protein sources in feed for farmed fish. Cahiers Agricultures. 18, 103–111.
Métayer, S., Seiliez, I., Collin, A., Duchêne, S., Mercier, Y., Geraert, P.-A., Tesseraud, S., 2008. Mechanisms through which sulfur amino acids control protein metabolism and oxidative status. J. Nutr. Biochem. 19, 207–215.
Morales, A.E., Garciarejon, L., Delahiguera, M., 1990. Influence of handling and/or anesthesia on tress response in rainbow-trout - effects on liver primary metabolism. Comp. Biochem. Physiol. Physiol. 95, 87–93.
Morales, A.E., Perez-Jimenez, A., Hidalgo, M.C., Abellan, E., Cardenete, G., 2004. Oxidative stress and antioxidant defenses after prolonged starvation in *Dentex dentex* liver. Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. 139, 153–161.
Moreira, I.S., Peres, H., Couto, A., Enes, P., Oliva-Teles, A., 2008. Temperature and dietary carbohydrate level effects on performance and metabolic utilisation of diets in European sea bass (*Dicentrarchus labrax*) juveniles. Aquaculture 274, 153–160.
Nguyen, T.N., Davis, D.A., 2009. Re-evaluation of total sulphur amino acid requirement and determination of replacement value of cystine for methionine in semi-purified diets of juvenile Nile tilapia, *Oreochromis niloticus*. Aquac. Nutr. 15, 247–253.
NRC, N.R.C., 2011. Nutrient Requirements of Fish and Shrimp. The National Academies Press, Washigton, DC.
Ojopagogo, Y.A., Adewale, I.O., Afolayan, A., 2013. Preliminary studies on the renaturation of denatured catfish (*Clarias gariepinus*) glutathione transferase. Fish Physiol. Biochem. 39, 1657–1663.
Oliva-Teles, A., 2012. Nutrition and health of aquaculture fish. J. Fish Dis. 35, 83–108.
Oliva-Teles, A., Enes, P., Peres, H., 2015. Replacing fishmeal and fish oil in industrial aquafeeds for carnivorous species. In: Davies, A. (Ed.), Feed and Feeding Practices in Aquaculture. Woodhead publishing series in food science, technology and nutrition. Woodhead publishing, Oxford, pp. 203–233.
Park, G.S., Takeuchi, T., Yokoyama, M., Seikai, T., 2002. Optimal dietary taurine level for growth of juvenile Japanese flounder *Paralichthys olivaceus*. Fish. Sci. 68, 824–829.
Peres, M.H., Oliva-Teles, A., 1999. Influence of temperature on protein utilization in juvenile European seabass (*Dicentrarchus labrax*). Aquaculture 170, 337–348.
Peres, H., Oliva-Teles, A., 2005. Protein and energy metabolism of European seabass (*Dicentrarchus labrax*) juveniles and estimation of maintenance requirements. Fish Physiol. Biochem. 31, 23–31.
Peres, H., Oliva-Teles, A., 2008. Lysine requirement and efficiency of lysine utilization in turbot (*Scophthalmus maximus*) juveniles. Aquaculture 275, 283–290.
Perez-Jimenez, A., Peres, H., Rubio, V.C., Oliva-Teles, A., 2012. The effect of hypoxia on intermediary metabolism and oxidative status in gilthead sea bream (*Sparus aurata*) fed on diets supplemented with methionine and white tea. Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. 155, 506–516.
Rodehutscord, M., Becker, A., Pack, M., Pfeffer, E., 1997. Response of rainbow trout (*Oncorhynchus mykiss*) to supplements of individual essential amino acids in a Semipurified diet, including an estimate of the maintenance requirement for essential amino acids. J. Nutr. 127, 1166–1175.
Sakai, T., Tabata, N., Watanabe, K., 1990. Bile pigments in the bile of marine fish: yellowtail, Red Sea bream, and flounder. Agric. Biol. Chem. 54, 2047–2053.
Salze, G.P., Davis, D.A., 2015. Taurine: a critical nutrient for future fish feeds. Aquaculture 437, 215–229.
Schuhmacher, A., Gropp, J.M., 1998. Carnitine - a vitamin for rainbow trout? J. Appl. Ichthyol. 14, 87–90.
Scott, M.D., Zuo, L., Lubin, B.H., Chiu, D.T., 1991. NADPH, not glutathione, status modulates oxidant sensitivity in normal and glucose-6-phosphate dehydrogenase-deficient erythrocytes. Blood 77, 2059–2064.
Seneviratne, C.K., Li, T., Khaper, N., Singal, P.K., 1999. Effects of methionine on endogenous antioxidants in the heart. Am. J. Phys. 277, H2124–H2128.
Spies, J.R., 1967. Determination of tryptophan in proteins. Anal. Chem. 39, 1412–1416.
Spitze, A.R., Wong, D.L., Rogers, Q.R., Fascetti, A.J., 2003. Taurine concentrations in animal feed ingredients; cooking influences taurine content. J. Anim. Physiol. Anim. Nutr. (Berl). 87, 251–262.
Tacon, A.G.J., Hasan, M.R., 2011. Demand and supply of feed ingredients for farmed fish and crustaceans. Trends and prospects. In: FAO (Ed.), Fisheries and Aquaculture Technical Paper.
Takagi, S., Murata, H., Goto, T., Ichiki, T., Munasinghe, D.M.S., Endo, M., Matsumoto, T., Sakurai, A., Hatate, H., Yoshida, T., Sakai, T., Yamashita, H., Ukawa, M., Kuramoto, T., 2005. The green liver syndrome is caused by taurine deficiency in yellowtail, *Seriola quinqueradiata* fed diets without fishmeal. Aquaculture Sci. 53, 279–290.
Takagi, S., Murata, H., Goto, T., Ichiki, T., Endo, M., Hatate, H., Yoshida, T., Sakai, T., Yamashita, H., Ukawa, M., 2006. Efficacy of taurine supplementation for preventing green liver syndrome and improving growth performance in yearling red sea bream *Pagrus major* fed low-fishmeal diet. Fish. Sci. 72, 1191–1199.
Takagi, S., Murata, H., Goto, T., Hatate, H., Endo, M., Yamashita, H., Miyatake, H., Ukawa, M., 2010. Necessity of dietary taurine supplementation for preventing green liver symptom and improving growth performance in yearling red sea bream *Pagrus major* fed nonfishmeal diets based on soy protein concentrate. Fish. Sci. 76, 119–130.
Takagi, S., Murata, H., Goto, T., Hatate, H., Endo, M., Yamashita, H., Miyatake, H., Ukawa, M., 2011. Role of taurine deficiency in inducing green liver symptom and effect of dietary taurine supplementation in improving growth in juvenile red sea bream *Pagrus major* fed non-fishmeal diets based on soy protein concentrate. Fish. Sci. 77, 235–244.
Thebault, H., Alliot, E., Pastoureaud, A., 1985. Quantitative methionine requirement of juvenile sea-bass (*Dicentrarchus labrax*). Aquaculture 50, 75–87.
Trenzado, C., Hidalgo, M.C., Garcia-Gallego, M., Morales, A.E., Furne, M., Domezain, A., Domezain, J., Sanz, A., 2006. Antioxidant enzymes and lipid peroxidation in sturgeon *Acipenser naccarii* and trout *Oncorhynchus mykiss*. A comparative study. Aquaculture 254, 758–767.
Tulli, F., Messina, M., Calligaris, M., Tibaldi, E., 2010. Response of European sea bass (*Dicentrarchus labrax*) to graded levels of methionine (total sulfur amino acids) in soya protein-based semi-purified diets. Br. J. Nutr. 104, 664–673.
Vandeputte, C., Guizon, I., Genestie-Denis, I., Vannier, B., Lorenzon, G., 1994. A microtiter plate assay for total glutathione and glutathione disulfide contents in cultured/isolated cells: performance study of a new miniaturized protocol. Cell Biol. Toxicol. 10, 415–421.
Weissbach, H., Resnick, L., Brot, N., 2005. Methionine sulfoxide reductases: history and cellular role in protecting against oxidative damage. Biochim. Biophys. Acta 1703, 203–212.
Wilson, R.P., 2002. Amino acids and proteins. In: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition, 3rd edition. Academic Press, pp. 143–179.
Wu, G.Y., Fang, Y.Z., Yang, S., Lupton, J.R., Turner, N.D., 2004. Glutathione metabolism and its implications for health. J. Nutr. 134, 489–492.
Xiao, W.W., Feng, L., Kuang, S.Y., Liu, Y., Jiang, J., Jiang, W.D., Hu, K., Li, S.H., Tang, L., Zhou, X.Q., 2012. Lipid peroxidation, protein oxidant and antioxidant status of muscle and serum for juvenile Jian carp (*Cyprinus carpio* Var. Jian) fed grade levels of methionine hydroxy analogue. Aquac. Nutr. 18, 90–97.
Yokoyama, M., Takeuchi, T., Park, G.S., Nakazoe, J., 2001. Hepatic cysteinesulphinate decarboxylase activity in fish. Aquac. Res. 32, 216–220.

Chapter 5

General conclusions, final considerations and future prospects

5.1 General conclusions

- Gilthead seabream growth performance and feed utilization were not improved by dietary Gln supplementation,
- The fact that dietary Gln supplementation did not affect gilthead seabream whole-body and liver composition, hepatic and intestinal GSase, GlNase, ALAT, ASAT and GDH activities, suggests that additional Gln was funnelled towards physiological purposes other than protein synthesis and AA catabolism.
- The dietary Gln surplus seemed to be used at the antioxidant defence level. This is supported by the increased tGSH and GSH levels observed in both liver and intestine, and by the enhanced SOD activity and GSSG content in the intestine of fish fed Gln supplemented diets. In the intestine, Gln supplementation also decreased GPX activity, supposedly as result of the enhanced ROS scavenging action of GSH, as supported by the increased GSSG content. Interestingly, these effects were more significant for the lower dietary Gln supplementation level (0.5%) and decreased with further Gln supplementation, indicating that other pathways involving Gln must have been enhanced in fish fed the higher Gln supplementation levels.
- As expected, gilthead seabream liver presented higher ALAT, ASAT, GDH and GSase activities than the intestine, as the liver is the major organ for AA conversion and metabolism. The intestine showed higher GlNase activity and lower GSase / GlNase ratio than the liver, which is in agreement with the high use of Gln as energy source in the intestine.
- As anticipated, growth, feed utilization, and nitrogen balance of gilthead sea bream were not affected by dietary Arg levels above established requirement. Despite the dietary Arg surplus and different Arg-Lys ratios tested no adverse effects to fish performance and whole-body composition were evidenced, suggesting that, within the levels tested, no toxic effect due to an Arg excess or Arg-Lys antagonism occurred.
- Similarly to what was observed with Gln supplementation, dietary Arg modulated gilthead sea bream enzymatic antioxidant response, reducing GPX activity in the liver and the intestine, and increasing SOD activity in the intestine, while maintaining the overall oxidative damage in both tissues.

- Data of the Gln and Arg supplementation studies evidenced some differences between liver and intestine main antioxidant defence mechanisms in gilthead sea bream, corroborating previous reports for other fish species (Trenzado *et al*., 2006; Castro *et al*., 2015). The intestine was more susceptible to oxidative damage, having a 2-fold higher LPO values than the liver, higher GSSG content, and higher GPX and GR activities. On the other hand, the liver presented higher G6PDH and CAT activities and tGSH and GSH contents. SOD activity values were similar in both organs. These differences reveal that, compared to the liver, the intestine antioxidant defence was more dependent on GSH provision.

- Dietary Gln and Arg supplementation failed to modify intestinal Gln and Arg uptake capacity. This is not surprising, given that distinct AA share the same brush border membrane transporters; are subject to cross-inhibition and induction of transporters; and usually a specific AA is not an effective inducer of its main transporter.

- European sea bass juveniles were shown to cope well with a high-plant-protein diet containing only 17.5% of the protein from fish origin (8% FM plus 2% soluble fish protein concentrate, on a dietary dry matter basis) and a Met level 12% below the establish requirement without compromising growth, feed and nitrogen utilisation.

- As the diets had a dietary total sulphur AA (TSAA) level (1.36% of the diet) above the establish requirement (TSAA 1.2-1.3% of the diet; Tulli *et al.,* 2010), some Met sparing must have occurred by reducing its transsulfuration into Cys. This seems to indicate that dietary Met requirements may be over-estimated and further studies to fine tune such requirements should be performed, using diets with an adequate TSAA level and an adequate Cys level, thus allowing to better establish specific Met requirements, without considering the need of Met to synthetize Cys.

- European sea bass juveniles performed adequately with the high-plant-protein diet without Tau supplementation. Moreover, supplementing the diet with 1% Tau did not improve fish performance, making it plausible to consider that European sea bass juveniles are capable of synthesising Tau at a sufficient rate to meet requirements.

- European sea bass showed higher CAT and SOD activities and lower tGSH, GSH, and GSSG contents in the intestine than in the liver. This might have been

caused by an increase of ROS generation in the intestine as a consequence of feeding fish a high-plant-protein diet. Still, the LPO values were less than 2-fold higher in the intestine than in the liver, as it is usually observed, suggesting that intestinal oxidative damage was kept at normal levels.

- As expected, dietary Met supplementation increased tGSH and GSH contents, although this effect was only noticed in the intestine. Since the liver is the main GSH producer and storage organ, and is also responsible for supplying the intestine with GSH through the plasma (Wu *et al*., 2004), it is possible that the increased intestinal GSH content was originated in the liver.

- Dietary Met and Tau supplementation affected differently the antioxidant enzymes activity in the liver: Met supplementation increased GPX and CAT activities, while Tau supplementation decreased GPX activity and increased or decreased CAT activity in the low and high dietary Met level, respectively.

- The fact that dietary Tau supplementation decreased tGSH, GSH, and GSSG contents in the intestine, without altering OSI and LPO values, also supports the assumption that Tau exerts its antioxidant action by avoiding ROS generation.

- In the intestine, both Met and Tau supplementation to diets with low Tau or Met levels, respectively, decreased GPX and GR activities, without affecting LPO values. These results indicate that fish fed the lower dietary Met and Tau level needed to increase its intestinal antioxidant enzymatic activity to avoid oxidative damage. This increased antioxidant enzyme activity was no longer required under adequate Met or Tau dietary provision, further attesting the important antioxidant role of these AA.

5.2 Final considerations

Evidence exist that dietary Gln or Arg supplementation improves growth performance and feed utilization of some fish species (Yan and Qiu-Zhou, 2006; Cheng *et al*., 2011; Qiyou *et al*., 2011; Cheng *et al*., 2012; Andersen *et al*., 2013; Pohlenz *et al*., 2013, 2014). However, such effects were not observed in other species (Tibaldi *et al*., 1994; Alam *et al*., 2002; Luo *et al.,* 2007; Tulli *et al*., 2007; Cheng *et al*., 2011; Pohlenz *et al*., 2012a; Zhou *et al*., 2012a; Andersen *et al*., 2014), including gilthead sea bream (this thesis), which could be indicative of species specific physiological differences.

This thesis also showed that European sea bass fed a plant-protein based diet (82.5% of the protein from plant origin) with a Met level 12% below the established requirement (Tulli *et al.,* 2010), grew the same as fish fed Met and Tau supplemented diets. Present results also provide further evidences that European sea bass may be produced with diets almost devoid of FM (90-95% protein replacement), without Tau supplementation (Kaushik *et al*., 2004; Torrecillas *et al*., 2017). Accordingly to other fish species, this study showed that it is possible to reduce dietary Met level below estimated requirement level, provided that diets have an adequate TSAA level (Harding *et al*., 1977; Griffin *et al*., 1994; Goff and Gatlin, 2004; Nguyen and Davis, 2009; Abidi and Khan, 2011; Farhat and Khan, 2014; Klatt *et al*., 2016; Poppi *et al*., 2017). Contrarily to what was observed in other marine fish species, Tau supplementation to plant-based diets did not ameliorate fish growth performance (Park *et al*., 2002; Kim *et al*., 2005; Matsunari *et al*., 2005; Lunger *et al*., 2007; Chatzifotis *et al*., 2008; Takagi *et al*., 2006; Takagi *et al*., 2008; Takagi *et al*., 2010; Enterria *et al*., 2011; Lim *et al*., 2013; Jirsa *et al*., 2014; Khaoian *et al*., 2014; Hien *et al.,* 2015; Wu *et al*., 2015; Koven *et al*., 2016).

These seemingly contradictory results, between studies or fish species, regarding dietary Gln, Arg, or Tau supplementation capacity to enhance fish growth performance and feed utilization may be due to different feed formulations or to species specific differences in metabolic use of these AA and in the endogenous capacity to synthesize Tau. Given that the AA tested, as dietary supplements, also did not modify fish whole-body composition and AA metabolism (only analysed in Gln and Arg studies), it may be concluded that each AA surplus should have been driven to other physiological processes rather than energy production, protein synthesis, gluconeogenesis or lipogenesis. This assumption is, at least in part, corroborated by the effects of these AA in the antioxidant responses in both gilthead sea bream and European sea bass.

Interestingly, Gln and Arg supplementation had very similar effects on antioxidant enzymes activities, decreasing GPX activity in both liver and intestine, and increasing

SOD activity in the intestine. Additionally, and as expected given Gln role as GSH precursor, Gln supplementation enhanced liver and intestine tGSH and GSH levels. GSH was not measured in the Arg supplementation study, due to the absence of a direct Arg role in GSH production. However, the similarities in the antioxidant enzymes activities with dietary Gln and Arg, suggest that Arg may also have been converted to ornithine by arginase and then originate Glu, through ornithine transaminase. Thus, it is plausible to consider that the identical Gln and Arg effects on antioxidant enzymes activities might also imply the conversion of both AA to Glu, the direct precursor of GSH. To confirm this hypothesis, further studies should measure arginase, ornithine transaminase activities and glutathione synthetase activities and determine liver and intestine GSH contents.

Dietary Met was also shown to act as a GSH precursor in European sea bass, increasing tGSH and GSH contents in the intestine of fish fed the Met supplemented diets. Additionally, Met may have also acted as methyl donor for DNA methylation, increasing GPX and CAT activities in the liver (Seneviratne *et al*., 1999; Feng *et al*., 2011; Xiao *et al*., 2012).

Moreover, dietary Met supplementation modulated antioxidant enzymes activities in both liver and intestine without, however, affecting their oxidative damage, as it was also observed with Tau supplementation. Nonetheless, contrary to Met, Tau supplementation effects consisted mainly on a reduction of antioxidant enzymes activities in both liver and intestine, besides also reducing tGSH, GSH, and GSSG contents in the intestine. Although such effects may seem negative for fish health, the fact that the overall oxidative damage was maintained agrees with the reported Tau role in directly reducing ROS production (Jong *et al*., 2012; Salze and Davis, 2015), therefore avoiding an increase of GSH levels and antioxidant enzymes activities.

The higher GPX and GR activities observed in the intestine of European sea bass fed the unsupplemented diet, when compared to fish fed Met or Tau supplemented diets, seems to indicate that both Met and Tau supplementation decreased fish susceptibility to oxidative damage, thus reducing the need for a high GPX and GR activities. Additionally, the unusually higher SOD and CAT activities observed in the intestine when compared to the liver, in contrast to what is generally observed (Castro *et al*., 2015), is indicative of increased ROS generation in the intestine due to the use of high-plant-protein diets (Guerreiro *et al*., 2015). Given the increasing use of plant-feedstuffs based diets in aquafeeds, this aspect deserves further investigation.

Overall, this thesis demonstrated that dietary Gln and Arg supplementation in diets for gilthead sea bream or Met and Tau supplementation in diets for European sea bass

modulate fish antioxidant response. In addition, this work provides evidence that European sea bass can be produced on a diet with 82.5% of plant protein and a Met level 12% below the established requirement (0.8-0.9% of the diet; Tulli *et al.* 2010) without negative effects on growth performance.

5.3 Future prospects

Since Gln and Arg utilization are known to increase under malnutrition, stress or disease conditions (Lobley *et al*., 2001; Aragão *et al*., 2008; Costas *et al*., 2008; Wu *et al*., 2011), further studies with these AA should consider the use of more challenging diets and more stressful rearing conditions, besides evaluating these AA effects in fish immunobiology and intestinal structure and metabolism, particularly in the case of dietary Gln supplementation.

It would also be important to re-evaluate European sea bass dietary Met requirements using diets with adequate TSAA levels and practical Cys levels, and to evaluate dietary Tau requirements, in different development stages, due to the reported progressive increment of CSD activity (Yokoyama *et al*., 2001; Martinez *et al*., 2004; Kim *et al*., 2005), and taking into account the dietary concentration of its precursors (Cys or Met). Moreover, given Met and Tau roles in European sea bass antioxidant response, further studies are required to elucidate the mechanisms underlying such actions, namely: Met regulation of the antioxidant enzymes production, through the supply of methyl groups for DNA methylation (Seneviratne *et al*., 1999; Feng *et al*., 2011; Xiao *et al*., 2012); the formation of the “methionine sulfoxide reductase” (MSR) system that protects functional residues from ROS (Levine *et al*., 2000; Métayer *et al*., 2008); and Tau regulation of mitochondrial electron donors accumulation, which inhibits the diversion of electrons to oxygen, preventing superoxide formation (Jong *et al*., 2012; Salze and Davis, 2015).

Finally, since Gln (Lobley *et al*., 2001; Cheng *et al*., 2011, Cheng *et al*., 2012; Zhu *et al*., 2011; Pohlenz *et al*., 2012b; Hu *et al*., 2015; Zhang *et al*., 2017;), Arg (Buentello and Gatlin, 1999; Yue *et al*., 2015; Zhang *et al*., 2017), Tau (Salze and Davis, 2015) and Met (Kuang *et al*., 2012; Machado *et al*., 2015; Pan *et al*., 2016) have been reported to intervene in several aspects of fish immune response, it would also be important to access these AA potential to enhance fish disease resistance and to attenuate the oxidative damage induced by the inflammatory response (Kuang *et al*., 2012; Pan *et al*., 2016).

Chapter 6

References

Abidi, S.F., Khan, M.A., 2009. Dietary arginine requirement of fingerling Indian major carp, *Labeo rohita* (Hamilton) based on growth, nutrient retention efficiencies, RNA/DNA ratio and body composition. J. Appl. Ichthyol. 25, 707-714.

Abidi, S.F., Khan, M.A., 2011. Total sulphur amino acid requirement and cystine replacement value for fingerling rohu, *Labeo rohita*: effects on growth, nutrient retention and body composition. Aquacult. Nutr. 17, 583-594.

Ahearn, G.A., Behnke, R.D., Zonno, V., Storelli, C., 1992. Kinetic heterogeneity of Na-D-glucose cotransport in teleost gastrointestinal tract. Am. J. Physiol.-Reg. I. 263, 1018-1023.

Alam, M.S., Teshima, S.I., Ishikawa, M., Koshio, S., 2002. Effects of dietary arginine and lysine levels on growth performance and biochemical parameters of juvenile Japanese flounder *Paralichthys olivaceus*. Fish. Sci. 68, 509-516.

Alam, M.S., Watanabe, W.O., Myers, A.R., Rezek, T.C., Carroll, P.M., Longfellow, S., 2011. Effects of replacement of menhaden fish meal protein by solvent-extracted soybean meal protein supplemented with or without L-Methionine and L-Lysine in the diet of juvenile southern flounder. N. Am. J. Aquacult. 73, 350-359.

Alliot, E., Febvre, A., Metailler, R., Pastoureaud, A., 1974. Besoins nutritifs du bar (*Dicentrarchus labrax* L.). Etude du taux de protéine et du taux de lipid dans le régime, Actes de Colloque CNEXO, pp. 215-228.

Andersen, S.M., Holen, E., Aksnes, A., Ronnestad, I., Zerrahn, J.E., Espe, M., 2013. Dietary arginine affects energy metabolism through polyamine turnover in juvenile Atlantic salmon (*Salmo salar*). Br J Nutr. 110, 1968-1977.

Andersen, S.M., Holen, E., Aksnes, A., Rønnestad, I., Zerrahn, J.E., Espe, M., 2014. Adult Atlantic salmon (*Salmo salar* L.) adapts to long-term surplus dietary arginine supplementation. Aquacult. Nutr. 21, 355-363.

Anderson, P.M., 2001. Urea and glutamine synthesis: Environmental influences on nitrogen excretion, Fish Physiology. Academic Press, pp. 239-277.

Anderson, P.M., Walsh, P.J., 1995. Subcellular-localization and biochemical-properties of the enzymes of carbamoyl-phosphate and urea synthesis in the batrachoidid fishes opsanus-beta, opsanus-tau and porichthys-notatus. J. Exp. Biol. 198, 755-766.

Andoh, T., 2007. Amino acids are more important insulinotropins than glucose in a teleost fish, barfin flounder (*Verasper moseri*). Gen. Comp. Endocrinol. 151, 308-317.

Aragão, C., Corte-Real, J., Costas, B., Dinis, M.T., Conceição, L.E.C., 2008. Stress response and changes in amino acid requirements in Senegalese sole (*Solea senegalensis* Kaup 1858). Amino Acids. 34, 143-148.

ARRAINA - Advanced Research Initiatives for Nutrition & Aquaculture, 2015. Feed Ingredients in Aquaculture 1st Technical Booklet, pp. 28. Available on: http://www.arraina.eu/disclaimer/news/1387-arraina-1st-technical-booklet-published (accessed on 8th June 2016).

Aruoma, O.I., Halliwell, B., Hoey, B.M., Butler, J., 1988. The antioxidant action of taurine, hypotaurine and their metabolic precursors. Biochem. J. 256, 251-255.

Baker, D.H., 2006. Comparative species utilization and toxicity of sulfur amino acids. J. Nutr. 136, 1670-1675.

Bakke-McKellep, A.M., Nordrum, S., Krogdahl, A., Buddington, R.K., 2000. Absorption of glucose, amino acids, and dipeptides by the intestines of Atlantic salmon (*Salmo salar* L.). Fish Physiol. Biochem. 22, 33-44.

Ball, R.O., Urschel, K.L., Pencharz, P.B., 2007. Nutritional consequences of interspecies differences in arginine and lysine metabolism. J. Nutr. 137, 1626-1641.

Ballestrazzi, R., Lanari, D., Dagaro, E., Mion, A., 1994. The effect of dietary protein level and source on growth, body composition, total ammonia and reactive phosphate excretion of growing sea bass (*Dicentrarchus labrax*). Aquaculture. 127, 197-206.

Balocco, C., Bogé, G., Roche, H., 1993. Neutral amino acid transport by marine fish intestine: role of the side chain. J. Comp. Physiol. B. 163, 340-347.

Baños, N., Planas, J.V., Gutierrez, J., Navarro, I., 1999. Regulation of plasma insulin-like growth factor-I levels in brown trout (*Salmo trutta*). Comp. Biochem. Physiol. C Toxicol. Pharmacol. 124, 33-40.

Bañuelos-Vargas, I., López, L.M., Pérez-Jiménez, A., Peres, H., 2014. Effect of fishmeal replacement by soy protein concentrate with taurine supplementation on hepatic intermediary metabolism and antioxidant status of totoaba juveniles (*Totoaba macdonaldi*). Comp. Biochem. Physiol. B. 170, 18-25.

Basurco, B., Lovatelli, A. and García, B., 2011. Current status of Sparidae Aquaculture. In: Pavlidis M.A. and Mylonas C.C. (Eds.), Sparidae: Biology and aquaculture of gilthead sea bream and other species. Wiley-Blackwell, Oxford, pp. 1-50.

Belghit, I., Skiba-Cassy, S., Geurden, I., Dias, K., Surget, A., Kaushik, S., Panserat, S., Seiliez, I., 2014. Dietary methionine availability affects the main factors involved in muscle protein turnover in rainbow trout (*Oncorhynchus mykiss*). Br J Nutr. 112, 493-503.

Benedito-Palos, L., Ballester-Lozano, G.F., Simó, P., Karalazos, V., Ortiz, Á., Calduch-Giner, J., Pérez-Sánchez, J., 2016. Lasting effects of butyrate and low FM/FO diets on growth performance, blood haematology/biochemistry and molecular growth-related markers in gilthead sea bream (*Sparus aurata*). Aquaculture. 454, 8-18.

Benedito-Palos, L., Navarro, J.C., Bermejo-Nogales, A., Saera-Vila, A., Kaushik, S., Perez-Sanchez, J., 2009. The time course of fish oil wash-out follows a simple dilution model in gilthead sea bream (*Sparus aurata* L.) fed graded levels of vegetable oils. Aquaculture. 288, 98-105.

Berge, G.E., Bakke-McKellep, A.M., Lied, E., 1999. In vitro uptake and interaction between arginine and lysine in the intestine of Atlantic salmon (*Salmo salar*). Aquaculture. 179, 181-193.

Berge, G.E., Goodman, M., Espe, M., Lied, E., 2004. Intestinal absorption of amino acids in fish: kinetics and interaction of the in vitro uptake of l-methionine in Atlantic salmon (*Salmo salar* L.). Aquaculture. 229, 265-273.

Boge, G., Roche, H., Balocco, C., 2002. Amino acid transport by intestinal brush border vesicles of a marine fish, *Boops salpa*. Comp. Biochem. Physiol. B. 131, 19-26.

Bonaldo, A., Roem, A.J., Fagioli, P., Pecchini, A., Cipollini, I., Gatta, P.P., 2008. Influence of dietary levels of soybean meal on the performance and gut histology of gilthead sea bream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax* L.). Aquacult. Res. 39, 970-978.

Boujard, T., Gelineau, A., Coves, D., Corraze, G., Dutto, G., Gasset, E., Kaushik, S., 2004. Regulation of feed intake, growth, nutrient and energy utilisation in European sea bass (*Dicentrarchus labrax*) fed high fat diets. Aquaculture. 231, 529-545.

Brasse-Lagnel, C., Lavoinne, A., Husson, A., 2009. Control of mammalian gene expression by amino acids, especially glutamine. FEBS J. 276, 1826-1844.

Buddington, R.K., Diamond, J.M., 1987. Pyloric ceca of fish: a "new" absorptive organ. Am. J. Physiol. Gastrointest. Liver Physio. 252, G65-G76.

Buddington, R.K., Krogdahl, Å., Bakke McKellep, A.M., 1997. The intestines of carnivorous fish: structure and functions and the relations with diet. Acta Physiol. Scand. 161, Suppl 638, 67-80.

Buentello, J.A., Gatlin, D.M., 1999. Nitric oxide production in activated macrophages from channel catfish (*Ictalurus punctatus*): influence of dietary arginine and culture media. Aquaculture. 179, 513-521.

Buentello, J.A., Gatlin, D.M., 2000. The dietary arginine requirement of channel catfish (*Ictalurus punctatus*) is influenced by endogenous synthesis of arginine from glutamic acid. Aquaculture. 188, 311-321.

Buentello, J.A., Gatlin, D.M., 2001. Effects of elevated dietary arginine on resistance of channel catfish to exposure to *Edwardsiella ictaluri*. J. Aquat. Anim. Health. 13, 194-201.

Buentello, J.A., Reyes-Becerril, M., Romero-Geraldo, M.D., Ascencio-Valle, F.D., 2007. Effects of dietary arginine on hematological parameters and innate immune function of channel catfish. J. Aquat. Anim. Health. 19, 195-203.

Caballero-Solares, A., Viegas, I., Salgado, M.C., Siles, A.M., Sáez, A., Metón, I., Baanante, I.V., Fernández, F., 2015. Diets supplemented with glutamate or glutamine improve protein retention and modulate gene expression of key enzymes of hepatic metabolism in gilthead seabream (*Sparus aurata*) juveniles. Aquaculture. 444, 79-87.

Carneiro, E.M., Latorraca, M.Q., Araujo, E., Beltra, M., Oliveras, M.J., Navarro, M., Berna, G., Bedoya, F.J., Velloso, L.A., Soria, B., Martin, F., 2009. Taurine supplementation modulates glucose homeostasis and islet function. J. Nutr. Biochem. 20, 503-511.

Cassano, G., Maffia, M., Ramires, P.A., Vilella, S., Storelli, C., 1990. Do neutral amino acids and their N-methylated analogues share the same Na-dependent carrier in brush-border membrane from the eel intestine? Comp. Biochem. Physiol. A. 96, 13-17.

Castro, C., Perez-Jimenez, A., Coutinho, F., Diaz-Rosales, P., Serra, C.A., Panserat, S., Corraze, G., Peres, H., Oliva-Teles, A., 2015. Dietary carbohydrate and lipid sources affect differently the oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles. Brit. J. Nutr. 114, 1584-1593.

Chatzifotis, S., Polemitou, I., Divanach, P., Antonopouiou, E., 2008. Effect of dietary taurine supplementation on growth performance and bile salt activated lipase activity of common dentex, *Dentex dentex*, fed a fish meal/soy protein concentrate-based diet. Aquaculture. 275, 201-208.

Chen, J., Zhou, X.-q., Feng, L., Liu, Y., Jiang, J., 2009. Effects of glutamine on hydrogen peroxide-induced oxidative damage in intestinal epithelial cells of Jian carp (*Cyprinus carpio* var. Jian). Aquaculture. 288, 285-289.

Cheng, Z.Y., Buentello, A., Gatlin, D.M., 2011. Effects of dietary arginine and glutamine on growth performance, immune responses and intestinal structure of red drum, *Sciaenops ocellatus*. Aquaculture. 319, 247-252.

Cheng, Z.Y., Gatlin, D.M., Buentello, A., 2012. Dietary supplementation of arginine and/or glutamine influences growth performance, immune responses and intestinal morphology of hybrid striped bass (*Morone chrysops* x *Morone saxatilis*). Aquaculture. 362, 39-43.

Cho, C.Y., Kaushik, S., Woodward, B., 1992. Dietary arginine requirement of young rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. A. 102, 211-216.

Clements, K.D., Raubenheimer, D., 2006. Feeding and nutrition. In: Evans, D.H., Claiborne, J.B. (Eds.), The physiology of fishes, 3rd Edition. CRC Press, New York, pp. 47-82.

Collie, N.L., Ferraris, R.P., 1995. Nutrient fluxes and regulation in fish intestine. In: Mommsen, H.a. (Ed.), Biochemestry and molecular biology of fishes. Elsevier Science, Amsterdam, pp. 221-239.

Costas, B., Aragão, C., Mancera, J.M., Dinis, M.T., Conceição, L.E.C., 2008. High stocking density induces crowding stress and affects amino acid metabolism in Senegalese sole *Solea senegalensis* (Kaup 1858) juveniles. Aquacult. Res. 39, 1-9.

Coutinho, F., Peres, H., Guerreiro, I., Pousão-Ferreira, P., Oliva-Teles, A., 2012. Dietary protein requirement of sharpsnout sea bream (*Diplodus puntazzo*, Cetti 1777) juveniles. Aquaculture. 356-57, 391-397.

Couto, A., Kortner, T.M., Penn, M., Bakke, A.M., Krogdahl, Å., Oliva-Teles, A., 2014. Effects of dietary phytosterols and soy saponins on growth, feed utilization efficiency and intestinal integrity of gilthead sea bream (*Sparus aurata*) juveniles. Aquaculture. 432, 295-303.

Couto, A., Kortner, T.M., Penn, M., Østby, G., Bakke, A.M., Krogdahl, Å., Oliva-Teles, A., 2015. Saponins and phytosterols in diets for European sea bass (*Dicentrarchus labrax*) juveniles: effects on growth, intestinal morphology and physiology. Aquacult. Nutr. 21, 180-193.

Couto, A., Peres, H., Oliva-Teles, A., Enes, P., 2017. Nutritional value of whole cereal meals for European sea bass (*Dicentrarchus labrax*) juveniles. Aquaculture. 473, 128-134.

Coutteau, P., Vanstappen, G., Sorgeloos, P., 1996. A standard experimental diet for the study of fatty acid requirements of weaning and first ongrowing stages of the European sea bass *Dicentrarchus labrax* L - Comparison of Extruded and Extruded/Coated Diets. Arch. Anim. Nutr.-Arch. Tierernahr. 49, 49-59.

Cowey, C.B., 1995. Protein and amino acid requirements - a critique of methods. J. Appl. Ichthyol. 11, 199-204.

Cowey, C.B., Cho, C.Y., Sivak, J.G., Weerheim, J.A., Stuart, D.D., 1992. Methionine intake in rainbow trout (*Oncorhynchus mykiss*), relationship to cataract formation and the metabolism of methionine. J. Nutr. 122, 1154-1163.

Cowey, C.B., Luquet, P., 1983. Physiological basis of protein requirements of fishes. Critical analysis of allowances, protein metabolism and nutrition. I.N.R.A.,France, pp. 365-384.

Cowey, C.B., Sargent, J.R., 1979. Nutrition. In: W.S. Hoar, D.J.R., Brett, J.R. (Eds.), Fish Physiology. Academic Press, pp. 69.

Cowey, C.B., Walton, M.J., 1989. Intermediary metabolism. In: Halver, J.E. (Ed.), Fish Nutrition 2nd Ed. Academic Press, New York, NY, pp. 260-329.

Curi, R., Lagranha, C.J., Doi, S.Q., Sellitti, D.F., Procopio, J., Pithon-Curi, T.C., Corless, M., Newsholme, P., 2005. Molecular mechanisms of glutamine action. J. cell. physiol. 204, 392-401.

Dabrowski, K., Guderley, H., 2002. Intermediary metabolism. In: Halver, J.E., Hardy, R.W. (Eds.), Fish nutrition, 3rd Edition. Academic Press, San Diego, pp. 309-365.

De Francesco, M., Parisi, G., Perez-Sanchez, J., Gomez-Requeni, P., Medale, F., Kaushik, S.J., Mecatti, M., Poli, B.M., 2007. Effect of high-level fish meal replacement by plant proteins in gilthead sea bream (*Sparus aurata*) on growth and body/fillet quality traits. Aquacult. Nutr. 13, 361-372.

Dias, J., Alvarez, M.J., Diez, A., Arzel, J., Corraze, G., Bautista, J.M., Kaushik, S.J., 1998. Regulation of hepatic lipogenesis by dietary protein/energy in juvenile European seabass (*Dicentrarchus labrax*). Aquaculture. 161, 169-186.

Dias, J., Gomes, E.F., Kaushik, S.J., 1997. Improvement of feed intake through supplementation with an attractant mix in European seabass fed plant-protein rich diets. Aquat. Living Resour. 10, 385-389.

Drai, P., Albertini-Berhaut, J., Lafaurie, M., Sudaka, P., Giudicelli, J., 1990. Simultaneous preparation of basolateral and brush-border membrane vesicles from sea bass intestinal epithelium. Biochim. Biophys. Acta. 1022, 251-259.

Eilertson, C.D., Sheridan, M.A., 1995. Pancreatic somatostatin-14 and somatostatin-25 release in rainbow trout is stimulated by glucose and arginine. Am. J. Physiol. 269, R1017-1023.

Ellis, A.E., 2001. Innate host defense mechanisms of fish against viruses and bacteria. Dev. Comp. Immunol. 25, 827-839.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2009. Nutritional regulation of hepatic glucose metabolism in fish. Fish Physiol. Biochem. 35, 519-539.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2011. Dietary Carbohydrate Utilization by European Sea Bass (*Dicentrarchus labrax* L.) and Gilthead Sea Bream (*Sparus aurata* L.) Juveniles. Rev. Fish. Sci. 19, 201-215.

Engelking, L.R., 2015. Chapter 8 - Amino acid catabolism, textbook of veterinary physiological chemistry (3rd Edition). Academic Press, Boston, pp. 45-51.

Enterria, A., Slocum, M., Bengtson, D.A., Karayannakidis, P.D., Lee, C.M., 2011. Partial replacement of fish meal with plant protein sources singly and in combination in

diets for summer flounder, *Paralichthys dentatus*. J. World Aquacult. Soc. 42, 753-765.

Espe, M., Lemme, A., Petri, A., El-Mowafi, A., 2006. Can Atlantic salmon (*Salmo salar*) grow on diets devoid of fish meal? Aquaculture. 255, 255-262.

Espe, M., Rathore, R.M., Du, Z.Y., Liaset, B., El-Mowafi, A., 2010. Methionine limitation results in increased hepatic FAS activity, higher liver 18:1 to 18:0 fatty acid ratio and hepatic TAG accumulation in Atlantic salmon, *Salmo salar*. Amino Acids. 39, 449-460.

EUFOMA, 2016. European market observatory for fisheries and aquaculture 2016, The EU fish market 2016 Edition, European Commission, Directorate-General for Maritime Affairs and Fisheries, Director-General (Ed.), Brussels, pp. 86.

Fan, M.Z., Adeola, O., McBurney, M.I., Cheeseman, C.I., 1998. Kinetic analysis of l-glutamine transport into porcine jejunal enterocyte brush-border membrane vesicles. Comp. Biochem. Physiol. A. 121, 411-422.

FAO, 2005a. Cultured aquatic species information programme. *Sparus aurata*. FAO Fisheries and Aquaculture Department [online], Rome, Italy. Available on: http://www.fao.org/fishery/culturedspecies/Sparus_aurata/en (accessed on 8th June 2016).

FAO, 2005b. Cultured aquatic species information programme. *Dicentrarchus labrax*. FAO Fisheries and Aquaculture Department [online], Rome, Italy. Available on: http://www.fao.org/fishery/culturedspecies/Dicentrarchus_labrax/en (accessed on 8th June 2016).

FAO, 2015. Regional review on status and trends in aquaculture development in Europe 2015. Food and Agriculture Organization of the United Nations (Ed.), Rome, pp. 41.

FAO, 2016. The state of world fisheries and aquaculture 2016. Food and Agriculture Organization of the United Nations (Ed.), Rome, pp. 190.

Farhat, Khan, M.A., 2014. Total sulfur amino acid requirement and cystine replacement value for fingerling stinging catfish, *Heteropneustes fossilis* (Bloch). Aquaculture. 426, 270-281.

Fauconneau, B., Arnal, M., 1985. In vivo protein synthesis in different tissues and the whole body of rainbow trout (*Salmo gairdnerii* R.). Influence of environmental temperature. Comp. Biochem. Physiol. A. 82, 179-187.

FEAP, 2016. European aquaculture production report 2005-2014. Belgium, pp. 54.

Feidantsis, K., Kaitetzidou, E., Mavrogiannis, N., Michaelidis, B., Kotzamanis, Y., Antonopoulou, E., 2014. Effect of taurine-enriched diets on the Hsp expression,

MAPK activation and the antioxidant defence of the European sea bass (*Dicentrarchus labrax*). Aquacult. Nutr. 20, 431-442.

Ferraris, R.P., Ahearn, G.A., 1984. Sugar and amino acid transport in fish intestine. Comp. Biochem. Physiol. A. 77, 397-413.

Ferraris, R.P., Diamond, J.M., 1989. Specific Regulation of Intestinal Nutrient Transporters by their Dietary Substrates. Annu. Rev. Physiol. 51, 125-141.

Figueiredo-Silva, C., Lemme, A., Sangsue, D., Kiriratnikom, S., 2015. Effect of DL-methionine supplementation on the success of almost total replacement of fish meal with soybean meal in diets for hybrid tilapia (*Oreochromis niloticus* × *Oreochromis mossambicus*). Aquacult. Nutr. 21, 234-241.

FishBase, 2017a. Available on: http://www.fishbase.org/summary/1164 (accessed on 8th June 2016).

FishBase, 2017b. Available on: http://www.fishbase.org/summary/63 (accessed on 8th June 2016).

Fisheries-EU, 2017a. Available on: https://ec.europa.eu/fisheries/marine_species/farmed_fish_and_shellfish/ (accessed on 8th June 2016).

Fournier, V., Gouillou Coustans, M.F., Metailler, R., Vachot, C., Guedes, M.J., Tulli, F., Oliva-Teles, A., Tibaldi, E., Kaushik, S.J., 2002. Protein and arginine requirements for maintenance and nitrogen gain in four teleosts. Brit. J. Nutr. 87, 459-469.

Fournier, V., Gouillou-Coustans, M.F., Metailler, R., Vachot, C., Moriceau, J., Le Delliou, H., Huelvan, C., Desbruyeres, E., Kaushik, S.J., 2003. Excess dietary arginine affects urea excretion but does not improve N utilisation in rainbow trout *Oncorhynchus mykiss* and turbot *Psetta maxima*. Aquaculture. 217, 559-576.

Fournier, V., Huelvan, C., Desbruyeres, E., 2004. Incorporation of a mixture of plant feedstuffs as substitute for fish meal in diets of juvenile turbot (*Psetta maxima*). Aquaculture. 236, 451-465.

Gatlin, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W., Herman, E., Hu, G., Krogdahl, Å., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., J Souza, E., Stone, D., Wilson, R., Wurtele, E., 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquacult. Res. 38, 551-579.

Gaylord, T.G., Teague, A.M., Barrows, F.T., 2006. Taurine supplementation of all-plant protein diets for rainbow trout (*Oncorhynchus mykiss*). J. World Aquacult. Soc. 37, 509-517.

Glencross, B.D., Booth, M., Allan, G.L., 2007. A feed is only as good as its ingredients - a review of ingredient evaluation strategies for aquaculture feeds. Aquacult. Nutr. 13, 17-34.

Goff, J.B., Gatlin, D.M., 2004. Evaluation of different sulfur amino acid compounds in the diet of red drum, *Sciaenops ocellatus*, and sparing value of cystine for methionine. Aquaculture. 241, 465-477.

Gomez-Requeni, P., Mingarro, M., Calduch-Giner, J.A., Medale, F., Martin, S.A.M., Houlihan, D.F., Kaushik, S., Perez-Sanchez, J., 2004. Protein growth performance, amino acid utilisation and somatotropic axis responsiveness to fish meal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*). Aquaculture. 232, 493-510.

Gomez-Requeni, P., Mingarro, M., Kirchner, S., Calduch-Giner, J.A., Medale, F., Corraze, G., Panserat, S., Martin, S.A.M., Houlihan, D.F., Kaushik, S.J., Perez-Sanchez, J., 2003. Effects of dietary amino acid profile on growth performance, key metabolic enzymes and somatotropic axis responsiveness of gilthead sea bream (*Sparus aurata*). Aquaculture. 220, 749-767.

Goto, T., Takagi, S., Ichiki, T., Sakai, T., Endo, M., Yoshida, T., Ukawa, M., Murata, H., 2001. Studies on the green liver in cultured red sea bream fed low level and non-fish meal diets: Relationship between hepatic taurine and biliverdin levels. Fish. Sci. 67, 58-63.

Gouillou-Coustans, M.F., Fournier, V., Metailler, R., Vachot, C., Desbruyeres, E., Huelvan, C., Moriceau, J., Le Delliou, H., Kaushik, S.J., 2002. Dietary arginine degradation is a major pathway in ureagenesis in juvenile turbot (*Psetta maxima*). Comp. Biochem. Physiol. A. 132, 305-319.

Gouveia, A., Davies, S.J., 1998. Preliminary nutritional evaluation of pea seed meal (Pisum sativum) for juvenile European sea bass (*Dicentrarchus labrax*). Aquaculture. 166, 311-320.

Griffin, M.E., White, M.R., Brown, P.B., 1994. Total sulfur amino acid requirement and cysteine replacement value for juvenile hybrid striped bass (*Morone saxatilis* × *M. chrysops*). Comp. Biochem. Physiol. A. 108, 423-429.

Guerreiro, I., Couto, A., Pérez-Jiménez, A., Oliva-Teles, A., Enes, P., 2015. Gut morphology and hepatic oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles fed plant feedstuffs or fishmeal-based diets supplemented with short-chain fructo-oligosaccharides and xylo-oligosaccharides. Brit. J. Nutr. 114, 1975-1984.

Halliwell, B., Chirico, S., 1993. Lipid peroxidation: its mechanism, measurement, and significance. Am. J. Clin. Nutr. 57, 715-724.

Halliwell, B., Gutteridge, J.M.C., 2015. Free radicals in biology and medicine, 5th Edition. Oxford University Press, Croydon, pp. 944.

Halver, J.E., Hardy, R.W., 2002. Nutrient flow and retention, Fish nutrition, 3rd Edition. Elsevier, pp. 755.

Han, Y., Koshio, S., Jiang, Z., Ren, T., Ishikawa, M., Yokoyama, S., Gao, J., 2014. Interactive effects of dietary taurine and glutamine on growth performance, blood parameters and oxidative status of Japanese flounder *Paralichthys olivaceus*. Aquaculture. 434, 348-354.

Harding, D.E., Allen, O.W., Wilson, R.P., 1977. Sulfur amino-acid requirement of channel catfish - L-methionine and L-cystine. J. Nutr. 107, 2031-2035.

He, J.Y., Tian, L.X., Lemme, A., Gao, W., Yang, H.J., Niu, J., Liang, G.Y., Chen, P.F., Liu, Y.J., 2013. Methionine and lysine requirements for maintenance and efficiency of utilization for growth of two sizes of tilapia (*Oreochromis niloticus*). Aquacult. Nutr. 19, 629-640.

Henrique, M.M.F., Gomes, E.F., Gouillou-Coustans, M.F., Oliva-Teles, A., Davies, S.J., 1998. Influence of supplementation of practical diets with vitamin C on growth and response to hypoxic stress of seabream, *Sparus aurata*. Aquaculture. 161, 415-426.

Hidalgo, F., Alliot, E., 1988. Influence of water temperature on protein requirement and protein utilization in juvenile sea bass, *Dicentrarchus labrax*. Aquaculture. 72, 115-129.

Hien, T.T.T., Be, T.T., Lee, C.M., Bengtson, D.A., 2015. Development of formulated diets for snakehead (*Channa striata* and *Channa micropeltes*): Can phytase and taurine supplementation increase use of soybean meal to replace fish meal? Aquaculture. 448, 334-340.

Hu, K., Feng, L., Jiang, W., Liu, Y., Jiang, J., Li, S., Zhou, X., 2014. Oxidative damage repair by glutamine in fish enterocytes. Fish Physiol. Biochem. 40, 1437-1445.

Hu, K., Zhang, J.X., Feng, L., Jiang, W.D., Wu, P., Liu, Y., Jiang, J., Zhou, X.Q., 2015. Effect of dietary glutamine on growth performance, non-specific immunity, expression of cytokine genes, phosphorylation of target of rapamycin (TOR), and anti-oxidative system in spleen and head kidney of Jian carp (*Cyprinus carpio* var. Jian). Fish Physiol. Biochem. 41, 635-649.

Huxtable, R.J., 1992. Phisiological actions of taurine. Physiol. Rev. 72, 101-163.

IFFO, 2013. IFFO Positional Statement. Is aquaculture growth putting pressure on feed fish stocks? And is the growth of aquaculture being restricted by finite supplies of fishmeal and fish? Available on: http://www.iffo.net/position-paper/aquaculture-growth-putting-pressure-feed-fish (accessed on 8th June 2016).

Ip, Y.K., Chew, S.F., 2010. Ammonia production, excretion, toxicity, and defense in fish: A Review. Front. Physiol. 1, 1-20.

Jacobsen, J.G., Smith, L.H., 1968. Biochemistry and physiology of taurine and taurine derivatives. Physiol. Rev. 18.

Jiang, J., Zheng, T., Zhou, X.Q., Liu, Y., Feng, L., 2009. Influence of glutamine and vitamin E on growth and antioxidant capacity of fish enterocytes. Aquacult. Nutr. 15, 409-414.

Jirsa, D., Davis, D.A., Salze, G.P., Rhodes, M., Drawbridge, M., 2014. Taurine requirement for juvenile white seabass (*Atractoscion nobilis*) fed soy-based diets. Aquaculture. 422, 36-41.

Jobling, M., 1994. Fish bioenergetics. In: Fish and fisherires series 13, Chapman & Hall, pp. 309.

Jong, C.J., Azuma, J., Schaffer, S., 2012. Mechanism underlying the antioxidant activity of taurine: prevention of mitochondrial oxidant production. Amino Acids. 42, 2223-2232.

Jürss, K., Bastrop, R., 1995. Amino acid metabolism in fish. In: Hochachka, P.W., Mommsen, T.P. (Eds.), Biochemistry and Molecular Biology of Fishes. Elsevier, Amsterdam, pp. 159-189.

Kader, M.A., Bulbul, M., Koshio, S., Ishikawa, M., Yokoyama, S., Nguyen, B.T., Komilus, C.F., 2012. Effect of complete replacement of fishmeal by dehulled soybean meal with crude attractants supplementation in diets for red sea bream, *Pagrus major*. Aquaculture. 350, 109-116.

Kader, M.A., Koshio, S., Ishikawa, M., Yokoyama, S., Bulbul, M., 2010. Supplemental effects of some crude ingredients in improving nutritive values of low fishmeal diets for red sea bream, *Pagrus major*. Aquaculture. 308, 136-144.

Kalogeropoulos, N., Alexis, M., Henderson, R.J., 1992. Effect of dietary soybean and cod-liver oil levels on growth and body composition of gilthead bream (*Sparus aurata*). Aquaculture. 104, 293-308.

Karapanagiotidis, I.T., 2014. The re-authorization of non-ruminant processed animal proteins in European aqua feeds. Fish Aquac. J. 5, 1-3.

Kaushik, S.J., 1998. Whole body amino acid composition of European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and turbot (*Psetta maxima*) with an estimation of their IAA requirement profiles. Aquat. Living Resour. 11, 355-358.

Kaushik, S.J., Coves, D., Dutto, G., Blanc, D., 2004. Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus labrax*. Aquaculture. 230, 391-404.

Kaushik, S.J., Fauconneau, B., 1984. Effects of lysine administration on plasma arginine and on some nitrogenous catabolites in rainbow trout. Comp. Biochem. Physiol. A. 79, 459-462.

Kaushik, S.J., Fauconneau, B., Terrier, L., Gras, J., 1988. Arginine requirement and status assessed by different biochemical indices in rainbow trout (*Salmo gairdneri* R.). Aquaculture. 70, 75-95.

Kaushik, S.J., Seiliez, I., 2010. Protein and amino acid nutrition and metabolism in fish: current knowledge and future needs. Aquacult. Res. 41, 322-332.

Keembiyehetty, C.N., Gatlin, D.M., 1995. Evaluation of different sulfur compounds in the diet of juvenile sunshine bass (*Morone chrysops* ♀ × *M. saxatilis* ♂). Comp. Biochem. Physiol. A. 112, 155-159.

Khaoian, P., Nguyen, H.P., Ogita, Y., Fukada, H., Masumoto, T., 2014. Taurine supplementation and palm oil substitution in low-fish meal diets for young yellowtail *Seriola quinqueradiata*. Aquaculture. 420–421, 219-224.

Kim, S.K., Matsunari, H., Takeuchi, T., Yokoyama, M., Furuita, H., Murata, Y., Goto, T., 2008. Comparison of taurine biosynthesis ability between juveniles of Japanese flounder and common carp. Amino Acids. 35, 161-168.

Kim, S.-K., Takeuchi, T., Yokoyama, M., Murata, Y., Kaneniwa, M., Sakakura, Y., 2005. Effect of dietary taurine levels on growth and feeding behavior of juvenile Japanese flounder *Paralichthys olivaceus*. Aquaculture. 250, 765-774.

Kiron, V., 2012. Fish immune system and its nutritional modulation for preventive health care. Anim. Feed Sci. Technol. 173, 111-133.

Kissil, G.W., Cowey, C.B., Adron, J.W., Richards, R.H., 1981. Pyridoxine requirements of the gilthead bream, *Sparus aurata*. Aquaculture. 23, 243-255.

Kissil, G.W., Lupatsch, I., 2004. Successful replacement of fishmeal by plant proteins in diets for the gilthead seabream, *Sparus aurata* L. Isr. J. Aquac.-Bamidgeh. 56, 188-199.

Kissil, G.W., Lupatsch, I., Higgs, D.A., Hardy, R.W., 1997. Preliminary evaluation of rapeseed protein concentrate as an alternative to fish meal in diets for gilthead seabream (*Sparus aurata*). Isr. J. Aquacult-Bamid. 49, 135-143.

Klatt, S.F., von Danwitz, A., Hasler, M., Susenbeth, A., 2016. Determination of the lower and upper critical concentration of Methionine + Cystine in diets of juvenile turbot (*Psetta maxima*). Aquaculture. 452, 12-23.

Koch, J.F., Rawles, S.D., Webster, C.D., Cummins, V., Kobayashi, Y., Thompson, K.R., Gannam, A.L., Twibell, R.G., Hyde, N.M., 2016. Optimizing fish meal-free commercial diets for Nile tilapia, *Oreochromis niloticus*. Aquaculture. 452, 357-366.

Kokou, F., Rigos, G., Henry, M., Kentouri, M., Alexis, M., 2012. Growth performance, feed utilization and non-specific immune response of gilthead sea bream (*Sparus aurata* L.) fed graded levels of a bioprocessed soybean meal. Aquaculture. 364, 74-81.

Koven, W., Peduel, A., Gada, M., Nixon, O., Ucko, M., 2016. Taurine improves the performance of white grouper juveniles (*Epinephelus Aeneus*) fed a reduced fish meal diet. Aquaculture. 460, 8-14.

Krogdahl, A., Hemre, G.I., Mommsen, T.P., 2005. Carbohydrates in fish nutrition: digestion and absorption in postlarval stages. Aquacult. Nutr. 11, 103-122.

Krogdahl, A., Penn, M., Thorsen, J., Refstie, S., Bakke, A.M., 2010. Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. Aquacult. Res. 41, 333-344.

Kuang, S.Y., Xiao, W.W., Feng, L., Liu, Y., Jiang, J., Jiang, W.D., Hu, K., Li, S.H., Tang, L., Zhou, X.Q., 2012. Effects of graded levels of dietary methionine hydroxy analogue on immune response and antioxidant status of immune organs in juvenile Jian carp (*Cyprinus carpio* var. Jian). Fish Shellfish Immunol. 32, 629-636.

Lall, S.P., Dumas, A., 2015. Nutritional requirements of cultured fish: Formulating nutritionally adequate feeds. Davies, A. (Ed.), Feed and feeding practices in aquaculture. woodhead publishing series in food science, technology and nutrition. Woodhead Publishing, Oxford, pp. 53-109.

Lall, S.P., Kaushik, S.J., Lebail, P.Y., Keith, R., Anderson, J.S., Plisetskaya, E., 1994. Quantitative Arginine Requirement of Atlantic Salmon (*Salmo salar*) Reared in Sea Water. Aquaculture. 124, 13-25.

Lanari, D., D'Agaro, E., 2005. Alternative plant protein sources in sea bass diets. Ital. J. Anim. Sci. 4, 365-374.

Lanari, D., Poli, B.M., Ballestrazzi, R., Lupi, P., D'Agaro, E., Mecatti, M., 1999. The effects of dietary fat and NFE levels on growing European sea bass (*Dicentrarchus labrax* L.). Growth rate, body and fillet composition, carcass traits and nutrient retention efficiency. Aquaculture. 179, 351-364.

Lansard, M., Panserat, S., Plagnes-Juan, E., Dias, K., Seiliez, I., Skiba-Cassy, S., 2011. L-Leucine, L-Methionine, and L-Lysine are involved in the regulation of intermediary metabolism-related gene expression in rainbow trout hepatocytes. J. Nutr. 141, 75-80.

Levine, R.L., Moskovitz, J., Stadtman, E.R., 2000. Oxidation of methionine in proteins: Roles in antioxidant defense and cellular regulation. IUBMB Life. 50, 301-307.

Li, H.T., Feng, L., Jiang, W.D., Liu, Y., Jiang, J., Li, S.H., Zhou, X.Q., 2013. Oxidative stress parameters and anti-apoptotic response to hydroxyl radicals in fish erythrocytes: Protective effects of glutamine, alanine, citrulline and proline. Aquat. Toxicol. 126, 169-179.

Li, M., Lai, H., Li, Q., Gong, S., Wang, R., 2016. Effects of dietary taurine on growth, immunity and hyperammonemia in juvenile yellow catfish *Pelteobagrus fulvidraco* fed all-plant protein diets. Aquaculture. 450, 349-355.

Li, P., Mai, K.S., Trushenski, J., Wu, G.Y., 2009. New developments in fish amino acid nutrition: towards functional and environmentally oriented aquafeeds. Amino Acids. 37, 43-53.

Li, P., Yin, Y.L., Li, D., Kim, S.W., Wu, G.Y., 2007. Amino acids and immune function. Brit. J. Nutr. 98, 237-252.

Liang, H., Ren, M., Habte-Tsion, H.-M., Ge, X., Xie, J., Mi, H., Xi, B., Miao, L., Liu, B., Zhou, Q., Fang, W., 2016. Dietary arginine affects growth performance, plasma amino acid contents and gene expressions of the TOR signaling pathway in juvenile blunt snout bream, *Megalobrama amblycephala*. Aquaculture. 461, 1-8.

Lim, S.-J., Oh, D.-H., Khosravi, S., Cha, J.-H., Park, S.-H., Kim, K.-W., Lee, K.-J., 2013. Taurine is an essential nutrient for juvenile parrot fish *Oplegnathus fasciatus*. Aquaculture. 414-415, 274-279.

Lin, H., Tan, X., Zhou, C., Niu, J., Xia, D., Huang, Z., Wang, J., Wang, Y., 2015. Effect of dietary arginine levels on the growth performance, feed utilization, non-specific immune response and disease resistance of juvenile golden pompano *Trachinotus ovatus*. Aquaculture. 437, 382-389.

Lobley, G.E., Hoskin, S.O., McNeil, C.J., 2001. Glutamine in animal science and production. The journal of nutrition. 131, 2525-2531.

Lunger, A.N., McLean, E., Gaylord, T.G., Kuhn, D., Craig, S.R., 2007. Taurine supplementation to alternative dietary proteins used in fish meal replacement enhances growth of juvenile cobia (*Rachycentron canadum*). Aquaculture. 271, 401-410.

Luo, Z., Liu, Y.J., Mai, K.S., Tian, L.X., Tan, X.Y., Yang, H.J., 2007. Effects of dietary arginine levels on growth performance and body composition of juvenile grouper *Epinephelus coioides*. J. Appl. Ichthyol. 23, 252-257.

Luquet, P., Sabaut, J.J., 1974. Nutrition azotée et croissance chez la daurade et la truite, Colloque sur l'aquaculture. Actes des colloques. CNEXO, Brest.

Machado, M., Azeredo, R., Díaz-Rosales, P., Afonso, A., Peres, H., Oliva-Teles, A., Costas, B., 2015. Dietary tryptophan and methionine as modulators of European

seabass (*Dicentrarchus labrax*) immune status and inflammatory response. Fish Shellfish Immunol. 42, 353-362.

Maffia, M., Acierno, R., Cillo, E., Storelli, C., 1996. Na(+)-D-glucose cotransport by intestinal BBMVs of the Antarctic fish *Trematomus bernacchii*. Am. J. Physiol. Reg. I. 271, 1576-1583.

Mambrini, M., Roem, A.J., Carvedi, J.P., Lalles, J.P., Kaushik, S.J., 1999. Effects of replacing fish meal with soy protein concentrate and of DL-methionine supplementation in high-energy, extruded diets on the growth and nutrient utilization of rainbow trout, *Oncorhynchus mykiss*. J. Anim. Sci. 77, 2990-2999.

Marcouli, P., Alexis, M.N., Andriopoulou, A., LLiopoulou-Georgudaki, J., 2005. Amino acid nutrition of gilthead seabream *Sparus aurata* juveniles: preliminary results on dietary lysine and methionine requirements. Cah. Options Mediterr. 63, 67-71.

Marcouli, P.A., Alexis, M.N., Andriopoulou, A., Iliopoulou-Georgudaki, J., 2006. Dietary lysine requirement of juvenile gilthead seabream *Sparus aurata* L. Aquacult. Nutr. 12, 25-33.

Martinez, J.B., Chatzifotis, S., Divanach, P., Takeuchi, T., 2004. Effect of dietary taurine supplementation on growth performance and feed selection of sea bass *Dicentrarchus labrax* fry fed with demand-feeders. Fish. Sci. 70, 74-79.

Martinez-Alvarez, R.M., Morales, A.E., Sanz, A., 2005. Antioxidant defenses in fish: Biotic and abiotic factors. Rev. Fish Biol. Fish. 15, 75-88.

Martinez-Llorens, S., Monino, A.V., Vidal, A.T., Salvador, V.J.M., Torres, M.P., Cerda, M.J., 2007. Soybean meal as a protein source in gilthead sea bream (*Sparus aurata* L.) diets: effects on growth and nutrient utilization. Aquacult. Res. 38, 82-90.

Matsunari, H., Takeuchi, T., Takahashi, M., Mushiake, K., 2005. Effect of dietary taurine supplementation on growth performance of yellowtail juveniles *Seriola quinqueradiata*. Fish. Sci. 71, 1131-1135.

Medale, F., Kaushik, S., 2009. Protein sources in feed for farmed fish. Cahiers Agricultures. 18, 103-111.

Messina, M., Piccolo, G., Tulli, F., Messina, C.M., Cardinaletti, G., Tibaldi, E., 2013. Lipid composition and metabolism of European sea bass (*Dicentrarchus labrax* L.) fed diets containing wheat gluten and legume meals as substitutes for fish meal. Aquaculture. 376, 6-14.

Metailler, R., Aldrin, J.F., Messager, J.L., Mevel, G., Stephan, G., 1981. Feeding of European sea bass *Dicentrarchus labrax*: role of protein and energy source. J. World Maricul. Soc. 12, 117-118.

Métayer, S., Seiliez, I., Collin, A., Duchêne, S., Mercier, Y., Geraert, P.-A., Tesseraud, S., 2008. Mechanisms through which sulfur amino acids control protein metabolism and oxidative status. J. Nutr. Biochem. 19, 207-215.

Mommsen, T.P., Moon, T.W., Plisetskaya, E.M., 2001. Effects of arginine on pancreatic hormones and hepatic metabolism in rainbow trout. Physiol. Biochem. Zool. 74, 668-678.

Morales, A.E., Perez-Jimenez, A., Hidalgo, M.C., Abellan, E., Cardenete, G., 2004. Oxidative stress and antioxidant defenses after prolonged starvation in *Dentex dentex* liver. Comp. Biochem. Physiol. C. 139, 153-161.

Morris, P.C., Davies, S.J., 1995a. The requirement of the gilthead seabream (S*parus aurata* L) for nicotinic acid. Anim. Sci. 61, 437-443.

Morris, P.C., Davies, S.J., 1995b. Thiamin supplementation of diets containing varied lipid - carbohydrate ratio given to gilthead seabream (*Sparus Aurata* L). Anim. Sci. 61, 597-603.

Morris, S.M., 2007. Arginine metabolism: Boundaries of our knowledge. J. Nutr. 137, 1602-1609.

Mourente, G., Bell, J., Tocher, D., 2007. Does dietary tocopherol level affect fatty acid metabolism in fish? Fish Physiol. Biochem. 33, 269-280.

Mourente, G., Diaz-Salvago, E., Bell, J.G., Tocher, D.R., 2002. Increased activities of hepatic antioxidant defence enzymes in juvenile gilthead sea bream (*Sparus aurata* L.) fed dietary oxidised oil: attenuation by dietary vitamin E. Aquaculture. 214, 343-361.

Moyano, F.J., Cardenete, G., Higuera, M.d.l., 1991. Nutritive and metabolic utilization of proteins with high glutamic acid content by the rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. A. 100, 752-762.

Naylor, R.L., Hardy, R.W., Bureau, D.P., Chiu, A., Elliott, M., Farrell, A.P., Forster, I., Gatlin, D.M., Goldburg, R.J., Hua, K., Nichols, P.D., 2009. Feeding aquaculture in an era of finite resources. Proceedings of the national academy of sciences. 106, 15103-15110.

Neu, D., Boscolo, W., Zaminhan, M., Almeida, F., Sary, C., Furuya, W., 2016. Growth performance, biochemical responses, and skeletal muscle development of juvenile Nile tilapia, *Oreochromis niloticus*, fed with Increasing levels of arginine. J. World Aquacult. Soc. 47, 248-259.

Ngamsnae, P., De.Silva, S.S., Gunasekera, R.M., 1999. Arginine and phenylalanine requirement of juvenile silver perch *Bidyanus bidyanus* and validation of the use of body amino acid composition for estimating individual amino acid requirements. Aquacult. Nutr. 5, 173-180.

Nguyen, T.N., Davis, D.A., 2009. Re-evaluation of total sulphur amino acid requirement and determination of replacement value of cystine for methionine in semi-purified diets of juvenile Nile tilapia, *Oreochromis niloticus*. Aquacult. Nutr. 15, 247-253.

N.R.C., 1993. Nutrient requirements of fish. The National Academy Press, Washington DC., pp. 128.

N.R.C., 2011. Nutrient requirements of fish and shrimp. The National Academies Press, Washigton, DC., pp. 373.

Nwanna, L.C., Lemme, A., Metwally, A., Schwarz, F.J., 2012. Response of common carp (*Cyprinus carpio* L.) to supplemental DL-methionine and different feeding strategies. Aquaculture. 356-57, 365-370.

Oehme, M., Grammes, F., Takle, H., Zambonino-Infante, J.L., Refstie, S., Thomassen, M.S., Rorvik, K.A., Terjesen, B.F., 2010. Dietary supplementation of glutamate and arginine to Atlantic salmon (*Salmo salar* L.) increases growth during the first autumn in sea. Aquaculture. 310, 156-163.

Oliva-Teles, A., Enes, P., Peres, H., 2015. Replacing fishmeal and fish oil in industrial aquafeeds for carnivorous species. In: Davies, A. (Ed.), Feed and feeding practices in aquaculture. Woodhead publishing series in food science, technology and nutrition. Woodhead Publishing, Oxford, pp. 203-233.

Oliva-Teles, A., Pimentel-Rodrigues, A., 2004. Phosphorus requirement of European sea bass (*Dicentrarchus labrax* L.) juveniles. Aquacult. Res. 35, 636-642.

Olsen, R.L., Hasan, M.R., 2012. A limited supply of fishmeal: Impact on future increases in global aquaculture production. Trends Food Sci. Technol. 27, 120-128.

Omnes, M.H., Silva, F.C.P., Moriceau, J., Aguirre, P., Kaushik, S., Gatesoupe, F.J., 2015. Influence of lupin and rapeseed meals on the integrity of digestive tract and organs in gilthead seabream (*Sparus aurata* L.) and goldfish (*Carassius auratus* L.) juveniles. Aquacult. Nutr., 21, 223-233.

Ozcan, A., Ogun, M., 2015. Biochemistry of reactive oxygen and nitrogen species. In: Gowder, S.J.T. (Ed.), Basic principles and clinical significance of oxidative stress. In Tech, Croatia, pp. 37 -58.

Pan, F.-Y., Feng, L., Jiang, W.-D., Jiang, J., Wu, P., Kuang, S.-Y., Tang, L., Tang, W.-N., Zhang, Y.-A., Zhou, X.-Q., Liu, Y., 2016. Methionine hydroxy analogue enhanced fish immunity via modulation of NF-κB, TOR, MLCK, MAPKs and Nrf2 signaling in young grass carp (*Ctenopharyngodon idella*). Fish Shellfish Immunol. 56, 208-228.

Pan, M., Meng, Q., Choudry, H.A., Karinch, A.M., Lin, C., Souba, W.W., 2004. Stimulation of intestinal glutamine absorption in chronic metabolic acidosis. Surgery. 136, 127-134.

Panserat, S., Kaushik, S., 2002. Régulation nutritionelle du métabolisme glucidique chez les poissons: exemple de la truite arc-en-ciel (*Oncorhynchus mykiss*), faible utilisatrice des glucides alimentaires. INRA Productions Animales. 15, 109-117.

Park, G.S., Takeuchi, T., Yokoyama, M., Seikai, T., 2002. Optimal dietary taurine level for growth of juvenile Japanese flounder *Paralichthys olivaceus*. Fish. Sci. 68, 824-829.

Pereira, R.T., Rosa, P.V., Gatlin Iii, D.M., 2017. Glutamine and arginine in diets for Nile tilapia: Effects on growth, innate immune responses, plasma amino acid profiles and whole-body composition. Aquaculture. 473, 135-144.

Pereira, T.G., Oliva-Teles, A., 2002. Preliminary evaluation of pea seed meal in diets for gilthead sea bream (*Sparus aurata*) juveniles. Aquacult. Res. 33, 1183-1189.

Pereira, T.G., Oliva-Teles, A., 2003. Evaluation of corn gluten meal as a protein source in diets for gilthead sea bream (*Sparus aurata* L.) juveniles. Aquacult. Res. 34, 1111-1117.

Pereira, T.G., Oliva-Teles, A., 2004. Evaluation of micronized lupin seed meal as an alternative protein source in diets for gilthead sea bream *Sparus aurata* L. juveniles. Aquacult. Res. 35, 828-835.

Peres, H., Oliva-Teles, A., 2005. The effect of dietary protein replacement by crystalline amino acid on growth and nitrogen utilization of turbot *Scophthalmus maximus* juveniles. Aquaculture. 250, 755-764.

Peres, H., Oliva-Teles, A., 2006. Effect of the dietary essential to non-essential amino acid ratio on growth, feed utilization and nitrogen metabolism of European sea bass (*Dicentrarchus labrax*). Aquaculture. 256, 395-402.

Peres, H., Oliva-Teles, A., 2007. Effect of the dietary essential amino acid pattern on growth, feed utilization and nitrogen metabolism of European sea bass (*Dicentrarchus labrax*). Aquaculture. 267, 119-128.

Peres, H., Oliva-Teles, A., 2009. The optimum dietary essential amino acid profile for gilthead seabream (*Sparus aurata*) juveniles. Aquaculture. 296, 81-86.

Peres, M.H., Gonçalves, P., Oliva-Teles, A., 1999. Glucose tolerance in gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*). Aquaculture. 179, 415-423.

Peres, M.H., Oliva-Teles, A., 1999a. Influence of temperature on protein utilization in juvenile European seabass (*Dicentrarchus labrax*). Aquaculture. 170, 337-348.

Peres, M.H., Oliva-Teles, A., 1999b. Effect of dietary lipid level on growth performance and feed utilization by European sea bass juveniles (*Dicentrarchus labrax*). Aquaculture. 179, 325-334.

Pérez, L., Gonzalez, H., Jover, M., Fernández-Carmona, J., 1997. Growth of European sea bass fingerlings (*Dicentrarchus labrax*) fed extruded diets containing varying levels of protein, lipid and carbohydrate. Aquaculture. 156, 183-193.

Pérez-Jiménez, A., Hidalgo, M.C., Morales, A.E., Arizcun, M., Abellan, E., Cardenete, G., 2009. Antioxidant enzymatic defenses and oxidative damage in *Dentex dentex* fed on different dietary macronutrient levels. Comp. Biochem. Physiol. C. 150, 537-545.

Pérez-Jiménez, A., Peres, H., Oliva-Teles, A., 2014. Effective replacement of protein-bound amino acids by crystalline amino acids in Senegalese sole (*Solea senegalensis*) juveniles. Aquacult. Nutr. 20, 60-68.

Pérez-Jiménez, A., Peres, H., Rubio, V.C., Oliva-Teles, A., 2012. The effect of dietary methionine and white tea on oxidative status of gilthead sea bream (*Sparus aurata*). Brit. J. Nutr. 108, 1202-1209.

Pérez-Ruzafa, Á., Marcos, C., 2015. Ecology and Distribution of *Dicentrarchus labrax* (Linnaeus 1758). In: Vázquez, J.S., Muñoz-Cueto, J. (Eds.), Biology of European Sea Bass. CRC Press, Boca Raton, Florida, pp. 3-33.

Pimentel Rodrigues, A.M., Oliva-Teles, A., 2001. Phosphorus requirements of gilthead sea bream (*Sparus aurata* L.) juveniles. Aquacult. Res. 32 Suppl. 1, 157-161.

Plisetskaya, E.M., Buchelli-Narvaez, L.I., Hardy, R.W., Dickhoff, W.W., 1991. Effects of injected and dietary arginine on plasma insulin levels and growth of pacific salmon and rainbow trout. Comp. Biochem. Physiol. A. 98, 165.

Pohlenz, C., Buentello, A., Bakke, A.M., Gatlin, D.M., 2012a. Free dietary glutamine improves intestinal morphology and increases enterocyte migration rates, but has limited effects on plasma amino acid profile and growth performance of channel catfish *Ictalurus punctatus*. Aquaculture. 370, 32-39.

Pohlenz, C., Buentello, A., Helland, S.J., Gatlin, D.M., 2014. Effects of dietary arginine supplementation on growth, protein optimization and innate immune response of channel catfish *Ictalurus punctatus* (Rafinesque 1818). Aquacult. Res. 45, 491-500.

Pohlenz, C., Buentello, A., Miller, T., Small, B.C., MacKenzie, D.S., Gatlin, D.M., 2013. Effects of dietary arginine on endocrine growth factors of channel catfish, *Ictalurus punctatus*. Comp. Biochem. Physiol. A. 166, 215-221.

Pohlenz, C., Buentello, A., Mwangi, W., Gatlin, D.M., 2012b. Arginine and glutamine supplementation to culture media improves the performance of various channel catfish immune cells. Fish Shellfish Immunol. 32, 762-768.

Poppi, D.A., Moore, S.S., Glencross, B.D., 2017. Redefining the requirement for total sulfur amino acids in the diet of barramundi (*Lates calcarifer*) including assessment of the cystine replacement value. Aquaculture. 471, 213-222.

Pozo, A.d., Falcon, J., Sanchez-Vazquez, J., 2015. The Biological Clock and Dualism. In: Vázquez, J.S., Muñoz-Cueto, J. (Eds.), Biology of European Sea Bass. CRC Press, Boca Raton, Florida, pp. 34-57.

Qi, G., Ai, Q., Mai, K., Xu, W., Liufu, Z., Yun, B., Zhou, H., 2012. Effects of dietary taurine supplementation to a casein-based diet on growth performance and taurine distribution in two sizes of juvenile turbot (*Scophthalmus maximus* L.). Aquaculture. 358–359, 122-128.

Qiyou, X., Qing, Z., Hong, X., Chang'an, W., Dajiang, S., 2011. Dietary glutamine supplementation improves growth performance and intestinal digestion/absorption ability in young hybrid sturgeon (*Acipenser schrenckii* female x *Huso dauricus* male). J. Appl. Ichthyol. 27, 721-726.

Reeds, P.J., Burrin, D.G., Stoll, B., Jahoor, F., 2000. Intestinal glutamate metabolism. J. Nutr. 130, 978-982.

Refstie, S., Landsverk, T., Bakke-McKellep, A.M., Ringo, E., Sundby, A., Shearer, K.D., Krogdahl, Å., 2006. Digestive capacity, intestinal morphology, and microflora of 1-year and 2-year old Atlantic cod (*Gadus morhua*) fed standard or bioprocessed soybean meal. Aquaculture. 261, 269-284.

Ren, M., Liao, Y., Xie, J., Liu, B., Zhou, Q., Ge, X., Cui, H., Pan, L., Chen, R., 2013. Dietary arginine requirement of juvenile blunt snout bream, *Megalobrama amblycephala*. Aquaculture. 414–415, 229-234.

Reshkin, S.J., Vilella, S., Cassano, G., Ahearn, G.A., Storelli, C., 1988. Basolateral amino acid and glucose transport by the intestine of the teleost, *Anguilla anguilla*. Comp. Biochem. Physiol. A. 91, 779-788.

Rodehutscord, M., Jacobs, S., Pack, M., Pfeffer, E., 1995. Response of rainbow trout (*Oncorhynchus Mykiss*) growing from 50 to 170 g to supplements of either l-arginine or l-threonine in a semipurified diet. J. Nutr. 125, 970-975.

Rolland, M., Dalsgaard, J., Holm, J., Gómez-Requeni, P., Skov, P.V., 2015. Dietary methionine level affects growth performance and hepatic gene expression of GH–IGF system and protein turnover regulators in rainbow trout (*Oncorhynchus mykiss*) fed plant protein-based diets. Comp. Biochem. Physiol. B. 181, 33-41.

Rolland, M., Skov, P.V., Larsen, B.K., Holm, J., Gomez-Requeni, P., Dalsgaard, J., 2016. Increasing levels of dietary crystalline methionine affect plasma methionine profiles, ammonia excretion, and the expression of genes related to the hepatic

intermediary metabolism in rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. B. 198, 91-99.

Sabaut, J.J., Luquet, P., 1973. Nutritional requirements of the gilthead bream *Chrysophrys aurata*. Quantitative protein requirements. Mar. Biol. 18, 50-54.

Sakai, T., Tabata, N., Watanabe, K., 1990. Bile pigments in the bile of marine fish: yellowtail, red sea bream, and flounder. Agric. Biol. Chem. 54, 2047-2053.

Sala-Rabanal, M., Gallardo, M.A., Sánchez, J., Planas, J.M., 2004. Na-dependent-glucose transport by intestinal brush border membrane vesicles from gilthead sea bream (*Sparus aurata*). J. Membr. Biol. 201, 85-96.

Salloum, R.M., Souba, W.W., Fernandez, A., Stevens, B.R., 1990. Dietary modulation of small intestinal glutamine transport in intestinal brush border membrane vesicles of rats. J. Surg. Res. 48, 635-638.

Salze, G.P., Davis, D.A., 2015. Taurine: a critical nutrient for future fish feeds. Aquaculture. 437, 215-229.

Santiago, C.B., Lovell, R.T., 1988. Amino acid requirements for growth of Nile tilapia. J. Nutr. 118, 1540-1546.

Santigosa, E., Garcia-Meilan, I., Valentin, J.M., Perez-Sanchez, J., Medale, F., Kaushik, S., Gallardo, M.A., 2011. Modifications of intestinal nutrient absorption in response to dietary fish meal replacement by plant protein sources in sea bream (*Sparus aurata*) and rainbow trout (*Onchorynchus mykiss*). Aquaculture. 317, 146-154.

Santinha, P.J.M., Gomes, E.F.S., Coimbra, J.O., 1996. Effects of protein level of the diet on digestibility and growth of gilthead sea bream, *Sparus auratus* L. Aquacult. Nutr. 2, 81-87.

Santinha, P.J.M., Medale, F., Corraze, G., Gomes, E.F.S., 1999. Effects of the dietary protein: lipid ratio on growth and nutrient utilization in gilthead seabream (*Sparus aurata* L.). Aquacult. Nutr. 5, 147-156.

Sargent, J.R., Tocher, D.R., Bell, J.G., 2002. The lipids. In: Halver, J.E., Hardy, R.W. (Eds.), Fish nutrition, 3rd Edition. Academic Press, San Diego, pp. 181-257.

Schaffer, S.W., Azuma, J., Mozaffari, M., 2009. Role of antioxidant activity of taurine in diabetes. Can. J. Physiol. Pharm. 87, 91-99.

Schep, L.J., Tucker, I.G., Young, G., Butt, A.G., 1997. Regional permeability differences between the proximal and distal portions of the isolated salmonid posterior intestine. J. Comp. Physiol. B. 167, 370-377.

Schuhmacher, A., Gropp, J.M., 1998. Carnitine - a vitamin for rainbow trout? J. Appl. Ichthyol. 14, 87-90.

Seneviratne, C.K., Li, T., Khaper, N., Singal, P.K., 1999. Effects of methionine on endogenous antioxidants in the heart. Am. J. Physiol. 277, 2124-2128.

Shepherd, C.J., Jackson, A.J., 2013. Global fishmeal and fish-oil supply: inputs, outputs and marketsa. J. Fish Biol. 83, 1046-1066.

Simmons, L., Moccia, R.D., Bureau, D.P., Sivak, J.G., Herbert, K., 1999. Dietary methionine requirement of juvenile Arctic charr *Salvelinus alpinus* (L.). Aquacult. Nutr. 5, 93-100.

Singh, S., Khan, M.A., 2007. Dietary arginine requirement of fingerling hybrid Clarias (*Clarias gariepinus* x *Clarias macrocephalus*). Aquacult. Res. 38, 17-25.

Sink, T.D., Lochmann, R.T., 2007. Insulin response of largemouth bass to glucose, amino acid, and diet stimulation. N. Am. J. Aquacult. 69, 429-434.

Sitja-Bobadilla, A., Pena-Llopis, S., Gomez-Requeni, P., Medale, F., Kaushik, S., Perez-Sanchez, J., 2005. Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). Aquaculture. 249, 387-400.

Skalli, A., Robin, J.H., 2004. Requirement of n-3 long chain polyunsaturated fatty acids for European sea bass (*Dicentrarchus labrax*) juveniles: growth and fatty acid composition. Aquaculture. 240, 399-415.

Skiba-Cassy, S., Geurden, I., Panserat, S., Seiliez, I., 2016. Dietary methionine imbalance alters the transcriptional regulation of genes involved in glucose, lipid and amino acid metabolism in the liver of rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 454, 56-65.

Spitze, A.R., Wong, D.L., Rogers, Q.R., Fascetti, A.J., 2003. Taurine concentrations in animal feed ingredients; cooking influences taurine content. J. Anim. Physiol. Anim. Nutr. 87, 251-262.

Stein, E.D., Chang, S.D., Diamond, J.M., 1987. Comparison of different dietary amino acids as inducers of intestinal amino acid transport. Am. J. Physiol. 252, 626-635.

Storey, K.B., 1996. Oxidative stress: animal adaptations in nature. Braz. J. Med. Biol. Res. 29, 1715-1733.

Tacon, A., Metian, M., Hasan, M.R., 2009. Feed ingredients and fertilizers for farmed aquatic animals. In: FAO (Ed.), FAO Fisheries and Aquaculture Technical Paper, No. 540. FAO, Rome,pp. 209.

Tacon, A.G.J., Hasan, M.R., 2011. Demand and supply of feed ingredients for farmed fish and crustaceans. Trends and prospects. In: FAO (Ed.), FAO Fisheries and aquaculture technical paper, No. 564, FAO, Rome, pp. 88.

Takagi, S., Murata, H., Goto, T., Endo, M., Yamashita, H., Ukawa, M., 2008. Taurine is an essential nutrient for yellowtail *Seriola quinqueradiata* fed non-fish meal diets based on soy protein concentrate. Aquaculture. 280, 198-205.

Takagi, S., Murata, H., Goto, T., Hatate, H., Endo, M., Yamashita, H., Miyatake, H., Ukawa, M., 2010. Necessity of dietary taurine supplementation for preventing green liver symptom and improving growth performance in yearling red sea bream *Pagrus major* fed nonfishmeal diets based on soy protein concentrate. Fish. Sci. 76, 119-130.

Takagi, S., Murata, H., Goto, T., Ichiki, T., Endo, M., Hatate, H., Yoshida, T., Sakai, T., Yamashita, H., Ukawa, M., 2006. Efficacy of taurine supplementation for preventing green liver syndrome and improving growth performance in yearling red sea bream *Pagrus major* fed low-fishmeal diet. Fish. Sci. 72, 1191-1199.

Takagi, S., Murata, H., Goto, T., Ichiki, T., Munasinghe, D.M.S., Endo, M., Matsumoto, T., Sakurai, A., Hatate, H., Yoshida, T., Sakai, T., Yamashita, H., Ukawa, M., Kuramoto, T., 2005. The green liver syndrome is caused by taurine deficiency in yellowtail, *Seriola quinqueradiata* fed diets without fishmeal. Aquacult. Sci. 53, 279-290.

Thebault, H., Alliot, E., Pastoureaud, A., 1985. Quantitative methionine requirement of juvenile sea-bass (*Dicentrarchus labrax*). Aquaculture. 50, 75-87.

Thillart, G.V., Van Raaij, M., 1995. Chapter 3 Endogenous fuels; non-invasive versus invasive approaches. Biochem. Mol. Biol. Fish. 4, 33-63.

Tibaldi, E., Hakim, Y., Uni, Z., Tulli, F., de Francesco, M., Luzzana, U., Harpaz, S., 2006. Effects of the partial substitution of dietary fish meal by differently processed soybean meals on growth performance, nutrient digestibility and activity of intestinal brush border enzymes in the European sea bass (*Dicentrarchus labrax*). Aquaculture. 261, 182-193.

Tibaldi, E., Lanari, D., 1991. Optimal dietary lysine levels for growth and protein utilisation of fingerling sea bass (*Dicentrarchus labrax* L.) fed semipurified diets. Aquaculture. 95, 297-304.

Tibaldi, E., Tulli, F., 1999. Dietary threonine requirement of juvenile european sea bass (*Dicentrarchus labrax*). Aquaculture. 175, 155-166.

Tibaldi, E., Tulli, F., Lanari, D., 1994. Arginine requirement and effect of different dietary arginine and lysine levels for fingerling Sea Bass (*Dicentrarchus labrax*). Aquaculture. 127, 207-218.

Tibaldi, E., Tulli, F., Lanari, D., Pinosa, M., 1993. Quantitative tryptophan requirement of sea bass fingerlings, EAS sp. publ., pp. 482.

Tocher, D.R., Mourente, G., Van der Eecken, A., Evjemo, J.O., Diaz, E., Bell, J.G., Geurden, I., Lavens, P., Olsen, Y., 2002. Effects of dietary vitamin E on antioxidant defence mechanisms of juvenile turbot (*Scophthalmus maximus* L.), halibut (*Hippoglossus hippoglossus* L.) and sea bream (*Sparus aurata* L.). Aquacult. Nutr. 8, 195-207.

Todgham, A.E., Anderson, P.M., Wright, P.A., 2001. Effects of exercise on nitrogen excretion, carbamoyl phosphate synthetase III activity and related urea cycle enzymes in muscle and liver tissues of juvenile rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. A. 129, 527-539.

Torrecillas, S., Mompel, D., Caballero, M.J., Montero, D., Merrifield, D., Rodiles, A., Robaina, L., Zamorano, M.J., Karalazos, V., Kaushik, S., Izquierdo, M., 2017. Effect of fishmeal and fish oil replacement by vegetable meals and oils on gut health of European sea bass (*Dicentrarchus labrax*). Aquaculture. 468, 386-398.

Trenzado, C., Hidalgo, M.C., Garcia-Gallego, M., Morales, A.E., Furne, M., Domezain, A., Domezain, J., Sanz, A., 2006. Antioxidant enzymes and lipid peroxidation in sturgeon *Acipenser naccarii* and trout *Oncorhynchus mykiss*. A comparative study. Aquaculture. 254, 758-767.

Tu, Y., Xie, S., Han, D., Yang, Y., Jin, J., Zhu, X., 2015. Dietary arginine requirement for gibel carp (*Carassis auratus gibelio* var. CAS III) reduces with fish size from 50 g to 150 g associated with modulation of genes involved in TOR signaling pathway. Aquaculture. 449, 37-47.

Tulli, F., Messina, M., Calligaris, M., Tibaldi, E., 2010. Response of European sea bass (*Dicentrarchus labrax*) to graded levels of methionine (total sulfur amino acids) in soya protein-based semi-purified diets. Brit. J. Nutr. 104, 664-673.

Tulli, F., Vachot, C., Tibaldi, E., Fournier, V., Kaushik, S.J., 2007. Contribution of dietary arginine to nitrogen utilisation and excretion in juvenile sea bass (*Dicentrarchus labrax*) fed diets differing in protein source. Comp. Biochem. Physiol. A 147, 179-188.

Van Nguyen, M., Rønnestad, I., Buttle, L., Van Lai, H., Espe, M., 2014. Imbalanced lysine to arginine ratios reduced performance in juvenile cobia (*Rachycentron canadum*) fed high plant protein diets. Aquacult. Nutr. 20, 25-35.

Vega-Rubín de Celis, S., Rojas, P., Gómez-Requeni, P., Albalat, A., Gutiérrez, J., Médale, F., Kaushik, S.J., Navarro, I., Pérez-Sánchez, J., 2004. Nutritional assessment of somatolactin function in gilthead sea bream (*Sparus aurata*): concurrent changes in somatotropic axis and pancreatic hormones. Comp. Biochem. Physiol. A. 138, 533-542.

Vergara, J.M., Fernandez-Palacios, H., Robaina, L., Jauncey, K., Delahiguera, M., Izquierdo, M., 1996b. The effects of varying dietary protein level on the growth, feed efficiency, protein utilization and body composition of gilthead sea bream fry. Fish. Sci. 62, 620-623.

Vergara, J.M., Jauncey, K., 1993. Studies on the use of dietary energy by gilthead sea bream (*Sparus aurata* L.) juveniles. In: Fish nutrition in practice. Les colloques. INRA, pp. 453-458.

Vergara, J.M., Lopez-Calero, G., Robaina, L., Caballero, M.J., Montero, D., Izquierdo, M.S., Aksnes, A., 1999. Growth, feed utilization and body lipid content of gilthead seabream (*Sparus aurata*) fed increasing lipid levels and fish meals of different quality. Aquaculture. 179, 35-44.

Vergara, J.M., Robaina, L., Izquierdo, M., Delahiguera, M., 1996a. Protein sparing effect of lipids in diets for fingerlings of gilthead sea bream. Fish. Sci. 62, 624-628.

Verri, T., Romano, A., Barca, A., Kottra, G., Daniel, H., Storelli, C., 2010. Transport of di- and tripeptides in teleost fish intestine. Aquacult. Res. 41, 641-653.

Walsh, P.J., Mommsen, T.P., 2001. Evolutionary considerations of nitrogen metabolism and excretion. In: Patricia, W., Paul, A. (Eds.), Fish Physiology. Academic Press, pp. 1-30.

Walton, M.J., Cowey, C.B., 1982. Apects of intermediary metabolism in salmonid fish. Comp. Biochem. Physiol. B. 73, 59-79.

Wang, B., Liu, Y., Feng, L., Jiang, W.D., Kuang, S.Y., Jiang, J., Li, S.H., Tang, L., Zhou, X.Q., 2015. Effects of dietary arginine supplementation on growth performance, flesh quality, muscle antioxidant capacity and antioxidant-related signalling molecule expression in young grass carp (*Ctenopharyngodon idella*). Food Chem. 167, 91-99.

Wang, L., Xu, Q., Wang, C.a., Li, J., Chen, D., Zhao, Z., Luo, L., Du, X., 2016a. Effects of dietary α-ketoglutarate supplementation on the growth performance, glutamine synthesis and amino acid concentrations of juvenile hybrid sturgeon *Acipenser schrenckii* ♀ × *Acipenser baerii* ♂ fed high levels of soy protein concentrate. Anim. Feed Sci. Technol. 211, 199-207.

Wang, Q.C., He, G., Wang, X., Mai, K.S., Xu, W., Zhou, H.H., 2014. Dietary sulfur amino acid modulations of taurine biosynthesis in juvenile turbot (*Psetta maxima*). Aquaculture. 422, 141-145.

Wang, Z., Mai, K., Xu, W., Zhang, Y., Liu, Y., Ai, Q., 2016b. Dietary methionine level influences growth and lipid metabolism via GCN2 pathway in cobia (*Rachycentron canadum*). Aquaculture. 454, 148-156.

Watford, M., 2015. Glutamine and glutamate: Nonessential or essential amino acids? Animal Nutrition. 1, 119-122.

Wilson, R.P., 2002. Amino acids and proteins. In: Halver, J.E., Hardy, R.W. (Eds.), Fish nutrition, 3rd Edition. Academic Press, San Diego, pp. 143-179.

Wood, E.J., 2000. Lehninger's principles of biochemistry, 3rd Edition. D.L. Nelson and M.M. Cox (Eds.), Worth Press, New York, pp. 1152.

Wu, G., 2009. Amino acids: metabolism, functions, and nutrition. Amino Acids. 37, 1-17.

Wu, G., 2010. Functional amino acids in growth, reproduction, and health. Adv. Nutr. 1, 31-37.

Wu, G., 2013a. Protein digestion and absorption of peptides and amino acids. In: Wu, G. (Eds.), Amino Acids. CRC Press, Boca Raton, pp. 33-62.

Wu, G., 2013b. Functional amino acids in nutrition and health. Amino Acids. 45, 407-411.

Wu, G., 2014. Dietary requirements of synthesizable amino acids by animals: a paradigm shift in protein nutrition. J. Anim. Sci. Biotech. 5, 34.

Wu, G., Bazer, F., Davis, T., Kim, S., Li, P., Marc Rhoads, J., Carey Satterfield, M., Smith, S., Spencer, T., Yin, Y., 2009. Arginine metabolism and nutrition in growth, health and disease. Amino Acids. 37, 153-168.

Wu, G., Bazer, F.W., Johnson, G.A., Knabe, D.A., Burghardt, R.C., Spencer, T.E., Li, X.L., Wang, J.J., 2011. Triennial Growth Symposium: important roles for L-glutamine in swine nutrition and production. J. Anim. Sci. 89, 2017-2030.

Wu, G., W., B.F., Zhaolai, D., Defa, L., Junjun, W., Zhenlong, W., 2014. Amino acid nutrition in animals: protein synthesis and beyond. Annu. Rev. Anim. Biosci. 2, 387-417.

Wu, G.Y., Fang, Y.Z., Yang, S., Lupton, J.R., Turner, N.D., 2004. Glutathione metabolism and its implications for health. J. Nutr. 134, 489-492.

Wu, Y., Han, H., Qin, J., Wang, Y., 2015. Replacement of fishmeal by soy protein concentrate with taurine supplementation in diets for golden pompano (*Trachinotus ovatus*). Aquacult. Nutr. 21, 214-222.

Xi, P., Jiang, Z., Zheng, C., Lin, Y., Wu, G., 2011. Regulation of protein metabolism by glutamine: implications for nutrition and health. Front. Biosci. 16, 578-597.

Yan, L., Qiu-Zhou, X., 2006. Dietary glutamine supplementation improves structure and function of intestine of juvenile Jian carp (*Cyprinus carpio* var. Jian). Aquaculture. 256, 389-394.

Yokoyama, M., Takeuchi, T., Park, G.S., Nakazoe, J., 2001. Hepatic cysteinesulphinate decarboxylase activity in fish. Aquacult. Res. 32, 216-220.

Yue, Y., Zou, Z., Zhu, J., Li, D., Xiao, W., Han, J., Yang, H., 2015. Effects of dietary arginine on growth performance, feed utilization, haematological parameters and non-specific immune responses of juvenile Nile tilapia (*Oreochromis niloticus* L.). Aquacult. Res. 46, 1801-1809.

Zhang, K., Mai, K., Xu, W., Liufu, Z., Zhang, Y., Peng, M., Chen, J., Ai, Q., 2017. Effects of dietary arginine and glutamine on growth performance, nonspecific immunity, and disease resistance in relation to arginine catabolism in juvenile turbot (*Scophthalmus maximus* L.). Aquaculture. 468, Part 1, 246-254.

Zhou, F., Shao, Q.J., Xiao, J.X., Peng, X., Ngandzali, B.O., Sun, Z., Ng, W.K., 2011. Effects of dietary arginine and lysine levels on growth performance, nutrient utilization and tissue biochemical profile of black sea bream, *Acanthopagrus schlegelii*, fingerlings. Aquaculture. 319, 72-80.

Zhou, F., Zhou, J., Shao, Q., Wang, Y., Li, H., Gao, S., Hua, Y., 2012a. Effects of arginine-deficient and replete diets on growth performance, digestive enzyme activities and genes expression of black sea bream, *Acanthopagrus schlegelii*, juveniles. J. World Aquacult. Soc. 43, 828-839.

Zhou, Q., Jin, M., Elmada, Z.C., Liang, X., Mai, K., 2015. Growth, immune response and resistance to *Aeromonas hydrophila* of juvenile yellow catfish, *Pelteobagrus fulvidraco*, fed diets with different arginine levels. Aquaculture. 437, 84-91.

Zhou, Q.C., Wu, Z.H., Tan, B.P., Chi, S.Y., Yang, Q.H., 2006. Optimal dietary methionine requirement for juvenile cobia (*Rachycentron canadum*). Aquaculture. 258, 551-557.

Zhou, Q.-C., Zeng, W.-P., Wang, H.-L., Xie, F.-J., Tuo, W., Zheng, C.-Q., 2012b. Dietary arginine requirement of juvenile yellow grouper *Epinephelus awoara*. Aquaculture. 350–353, 175-182.

Zhu, Q., Xu, Q.Y., Xu, H., Wang, C.A., Sun, D.J., 2011. Dietary glutamine supplementation improves tissue antioxidant status and serum non-specific immunity of juvenile Hybrid sturgeon (*Acipenser schrenckii* female x *Huso dauricus* male). J. Appl. Ichthyol. 27, 715-720.